EVOLUTION OF A NOVEL GENE PAIR FROM A CANONICAL TOXIN-ANTITOXIN MODULE IN *ESCHERICHIA COLI*

By

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ABSTRACT OF THE THESIS

EVOLUTION OF A NOVEL GENE PAIR FROM A CANONICAL TOXIN-ANTITOXIN MODULE IN *ESCHERICHIA COLI*

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Free-living bacteria are continuously subjected to environmental stress. This stress can be in the form of a change in temperature, pH, osmolarity or nutritional starvation. Most bacterial species contain gene modules known as Toxin-Antitoxin (TA) systems that reversibly inhibit cellular growth in response to stress; thereby helping the cells cope with a changing environment. One mechanism that bacteria have developed to combat fluctuations in environmental temperature is the cold-shock response. This response helps exponentially growing cells buffer themselves against a downshift in temperature from their optimal growing temperature; typically a shift from 37°C to 15°C for *Escherichia coli* (*E. coli*). Cold-shock proteins (Csp) are synthesized at this time. Protein Y (PY), the protein product of gene *yfiA* in *E. coli* is suggested to be a cold-shock related protein. It prevents ribosomes from dissociation during cold-shock, and in stationary phase, thereby blocking translational elongation and inhibiting cell growth. This mechanism resembled that of a typical TA system toxin. We identified a small gene,

b2596, upstream of *yfiA* and propose that the *b2596-yfiA* module evolved from a true proteic TA system that functioned in cold-shock conditions; Protein X (PX), product of *b2596*, being the antitoxin and PY the toxin. The module still retains some of its TA system characteristics: both genes encode small proteins, have opposing charges and show sequence similarity to known TA genes. Also, like a true TA system *b2596*, the proposed antitoxin gene, precedes *yfiA*, the proposed toxin gene. However, we found that the two genes have independent transcriptional start sites. Also *b2596* encodes a leaderless mRNA with UUG start and thus we predict that it cannot be translated well in vivo. PY inhibits growth of *E. coli* cells and functions in helping the bacterial population to survive cold-shock. Our data suggest that *b2596* and *yfiA* have evolved from a canonical proteic TA module that was functional in cold shock. The two genes are now independent and responsive to cold shock.

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First and foremost I would like to thank Dr. Nancy Woychik for her help, support and guidance in my thesis project and beyond. Thank you for teaching me to be precise in analyzing data and concise in presenting it. Next I would like to thank Jennifer Hurley for sharing her knowledge, time and bench with me. Jenny, you were a very patient teacher, I learnt much more than laboratory techniques from you; thank you for it all. I would like to thank Dr. Andrew Vershon for his advice and support during the course of M.S and taking the time to be on my thesis committee. I would also like to thank Dr. Sangita Phadtare for her time and helpful comments and for being on my thesis committee. I would also like to thank the members of the Woychik lab: Lauren DeStefano, Mohan Lui, Christopher Mozdzierz, Rohini Roy, Francesca Rothenbacher, Jason Schifano and Jared Sharp. You have all taught and helped me at different junctures. I owe thanks to Meredith Prysak and Jennifer Hurley for their contribution to this project. I am grateful to our collaborators in the Inouye lab for their support and resources. I am glad I got the opportunity to be part of the Woychik lab; the past year has been great and I will always remember the crazy, amusing, melodic, frenzied and fun moments in lab.

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Introduction

Toxin-Antitoxin Systems

The term "toxin-antitoxin (TA) module" describes a pair of genes wherein one gene encodes an unstable antitoxin that inhibits, or reduces, the potentially lethal action of the gene encoded by the second gene, the toxin. The toxin helps the cell adjust the rates of DNA and protein synthesis in response to environmental changes. Under cues of stress the labile antitoxin degrades quickly. The toxin protein, no longer sequestered by the antitoxin, is now free to act upon the cell. The toxin slows cell growth to avoid cell death during stress conditions [Gerdes et al., 2005]. TA modules thus play an important role in a free-living bacterial cell's response to stress.

There are some general criteria that gene pairs have to meet to be labeled as a TA module. First, the two genes are adjacent to one another with the antitoxin almost always preceding the toxin. The open reading frames of these genes have a one or two base pair gap in between or overlap by a few base pairs. The genes encode relatively small proteins; about 80-100 amino acids in length. TA modules are transcribed as a bicistronic message, under the control of a single promoter but are translated separately. Second, it has been well established that TA systems rely on the difference in half life between the toxin product and its cognate antitoxin [Van Melderen et al., 1994]. Under normal (stress-free), growth conditions the genes products come together to form a stable complex, thereby blocking the toxic effect of the toxin. Of the pair, as stated earlier, the antitoxin in relatively unstable. Finally, the toxin-antitoxin protein complex binds to a

palindromic sequence located upstream of the coding region. This serves as a mode for autoregulation.

The labile antitoxins usually contain a DNA binding domain in the amino-terminus (N-terminal) region and a caboxy-terminus (C-terminal) toxin inhibiting domain. When the antitoxin is bound to the toxin, this C-terminal domain adopts an extended conformation which protects the antitoxin from proteolytic cleavage. Without the toxin, the C-terminal region remains unstructured and this is thought to signal degradation mechanisms [Buts et al., 2005]. Thus under normal growth conditions the toxin-antitoxin complex serves as a repressor of the TA operon [de Feyter et al., 1989; Magnuson et al., 1998: Gronlund et al., 1999]. When the bacteria are introduced to stress conditions the degradation of the antitoxin outpaces its synthesis and hence the toxin acts on the cell. Cells can escape the effects of the toxin if stress conditions are alleviated and the antitoxin is produced again. TA toxins function in stress survival and this differentiates them from more familiar toxins like anthrax and botulinum. The main function of a TA toxin is to impart a state of 'quasi-dormancy' to the cell until stress conditions pass [Gerdes et al, 2005].

Obligate-host associate parasites or organism or those that live in close association with other organisms (parasitically or symbiotically), have not retained TA loci. These organisms live in constant environments, not experiencing fluxes in nutritional availability, pH, temperature etc. In contrast most free-living bacteria have TA systems since they are subjected to an ever-changing environment [Pandey and Gerdes, 2005].

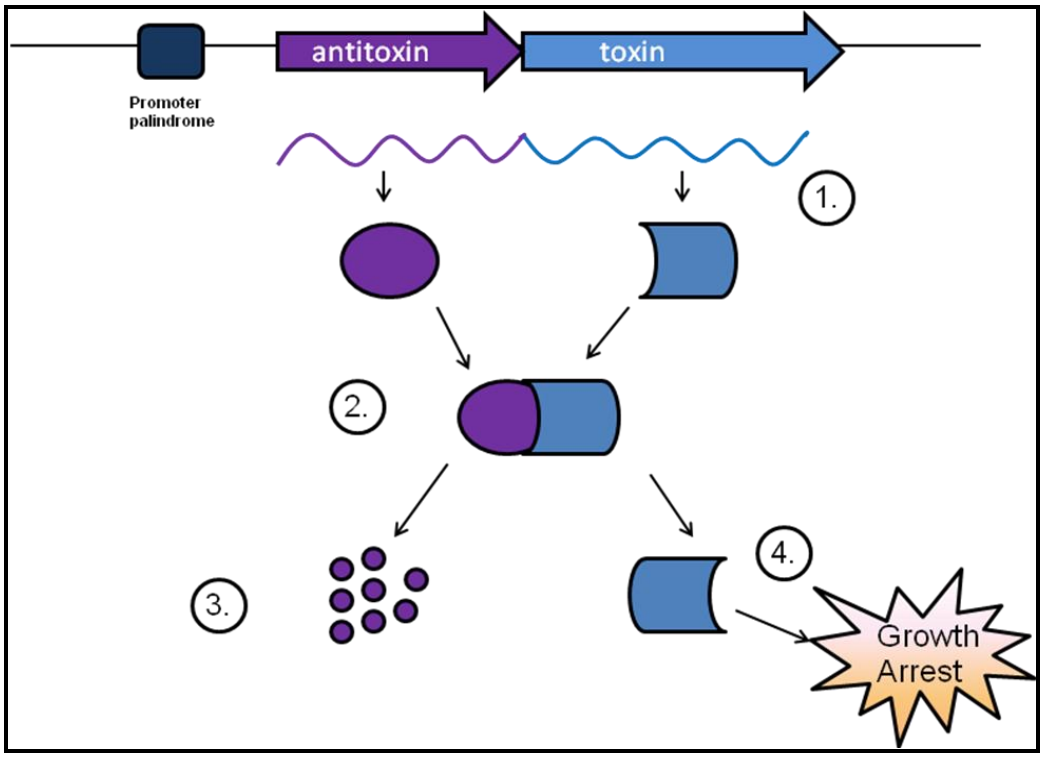


Figure 1: Proteic TA System Model: **1**. Toxin and Antitoxin genes are expressed as a bicistronic message; **2.** Toxin is sequestered by the Antitoxin and a stable complex is formed; **3**. Labile antitoxin degrades due to environmental stress and **4**. Toxin is free to act on the cell, causing growth arrest.

TA modules were first identified on bacterial plasmids and were characterized on their involvement in post-segregational killing (PSK), a plasmid maintenance mechanism [Gerdes et al., 1986; Ogura et al., 1983; Engelberg-Kulka et al., 1999]. These are grouped together as plasmid-based TA modules and are also known as 'Addiction Modules'. TA systems have also been identified in bacteriophages and prokaryotic chromosomes, including that of *Escherichia coli* (*E. coli*) (Table 2) [Pandey et al, 2005; Hayes, 2003; Gerdes et al., 2005].

Table 1: The TA gene families

TA Family	Toxin	Toxin target	Antitoxin
ccD	CcdB	Replication through DNA gyrase	CcdA
relBE	RelE	Translation through mRNA cleavage	RelB
parDE	ParE	Replication through DNA gyrase	ParD
higBA	HigB	Translation through mRNA cleavage	HigA
mazEF	MazF	Translation through mRNA cleavage	MazE
phd/doc	Doc	Translation via 30S ribosomal subunit	Phd
vapBC	VapC	Unknown	VapB
hipBA	HipB	Unknown	HipA

(Adapted from Gerdes et al., 2005)

Plasmid based TA modules and those found in bactriophages are grouped together as non-chromosomally encoded TA systems. There are eight typical, two-component, toxin-antitoxin gene families (Table 1) [Gerdes et al., 2005]. All families are found on plasmids and chromosomes. Genetic and structural analyses have revealed evolutionary relationships between several TA families [Buts et al., 2005].

E. coli Toxin-Antitoxin Systems

E. coli harbors many TA module families and they have been put into two broad categories: Non-chromosomally Encoded TA Systems and Chromosomal TA Systems. Table 2 lists the major families that these categories comprise.

Table 2: *E. coli* Toxin-Antitoxin Systems

TA Module	Toxin	Antitoxin	Location
ccD	*ccdB*	*ccdA*	Plasmid F
parDE	*parE*	*parD*	Plasmid TK2/RP4
phd/doc	*doc*	*phd*	Prophage1
mazEF	*mazE*	*mazF*	Chromosomal
chpBI/BK	*chpBI*	*chpBK*	
relBE	*relB*	*relE*	
yefM/yoeB	*yefM*	*yoeB*	
dinJ/yafQ	*dinJ*	*yafQ*	
hipBA	*hipB*	*hipA*	

, & : Denote different families

Non-chromosomally Encoded TA Systems

Plasmid-based TA systems were the first to be discovered over twenty years ago [Ogura and Hiraga, 1983]. These TA modules are involved in PSK [Gerdes et al., 1986]. On cell division, daughter cells that have not inherited a copy of the plasmid that expresses the TA module lose their ability to produce the antitoxin. Hence, after rapid degradation of residual antitoxin in the daughter cell the excess toxin is free to act in the cell. This provides a selection mechanism for plasmid maintenance in the cell [Brown and Shaw, 2003]. Since the cells depend on the plasmid to survive, by avoiding effects of

the toxin, these TA modules were termed as 'addiction modules' [Anantharaman and Aravind, 2003; Engelberg-Kulka et al., 1999].

Phd-doc **locus of bacteriophages P1**: This system resides on bacteriophage P1 and comprises the antitoxin gene *phd* (prevent host death) and the toxin gene *doc* (death on curing) [Lehneherr et al., 1993]. P1 lysogenizes in host cells as a stable P1 plasmid and is involved in PSK. Phd is more labile than its cognate toxin and is rapidly degraded by ClpXP serine protease [Lehnherr & Yarmolinsky, 1995]. The mechanism of Doc toxicity was discovered in our laboratory recently by Mohan Liu [Liu et al., 2008]. Doc associates with the 30S ribosomal subunit, disrupts translation, and leads to cell growth arrest [Liu et al, 2008]. The Phd-Doc complex also exerts autoregulatory effects by binding to a palindromic sequence upstream of its open reading frame [Magnuson et al., 1996]

Plasmid TA modules *ccdAB* and *parDE* : Ogura et al. discovered the first TA system, the *ccd* (control of cell death) locus on the F plasmid [Ogura et al., 1983; Engelberg-Kulka et al., 1999]. *ccdB* encodes the toxin and *ccdA* codes for the antitoxin. This addiction module causes cell death if absent in a progeny, due to the action of residual toxin in the cell [Gerdes et al., 1986]. When the toxin, ccdB is free it binds to subunit A of DNA gyrase and inhibits DNA replication and mRNA transcription [Miki et al., 1992].

parDE also operates via PSK and is found on a broad host-range plasmid RK2/RP4 [Engelberg-Kulka et al., 1999; Oberer et al., 2002]. ParD, the labile antitoxin , and ParE the toxin are directly involved in PSK when the host plasmid is lost from the

cell [Johnson et al., 1996]. The ParDE stable complex is a tetramer comprising two units of both the toxin and antitoxin. ParE, when unsequestered, targets DNA gyrase to inhibit replication [Engelberg-Kulka et al., 1999; Johnson et al., 1996]. The protease involved in the degradation of ParD is unknown.

Chromosomal TA modules: Chromosomally encoded TA systems are similar to the extra-chromosomal modules describe earlier. Their antitoxin component is labile, the toxin and antitoxin are regulated by a single promoter and form a stable complex under normal growth conditions. However, the toxins in chromosomal TA modules do not signal cell death when free. Their role is very different from toxins like Doc and ccdB which act in PSK. Chromosomally encoded TA toxins function to modulate the global levels of translation and replication during exposure to stress. These toxins are activated like their non-chromosomal counterparts, when freed from the antitoxin. They cause growth arrest in a cell and make it ‘quasi-dormant’ until the stressor has passed. They permit the cells to continue with normal growth hereafter. There are three main families in this category and Table 3 contains a brief description of the components of each family.

Table 3: Chromosomal-TA Modules

TA Module	Description
mazEF	Toxin: MazF ('ma-ze' means 'what is it?' in Hebrew) Antitoxin: MazE Mechanism: MazF disrupts translation via cleavage of mRNA at↓ACA
chpBI/BK	Toxin: chpBK Antitoxin: ChpBI Mechanism: ChpBK disrupts translation via cleavage of mRNA at either ACU↓, ACA↓ or ACG↓
relBE	Toxin: RelE Antitoxin: RelB Mechanism: RelE disrupts translation via specific cleavage of mRNA at ribosomal A site
yefM/yoeB	Toxin: YoeB Antitoxin: YefM Mechanism: YoeB disrupts translation via specific mRNA cleavage
dinJ/yafQ	Toxin: YafQ Antitoxin: DinJ (damage inducible gene family) Mechanism:YafQ disrupts translation via ribosome dependent cleavage of mRNA at in-frame AA↓A- G/A
hipBA	Toxin: HipA (high incidence of persistence) Antitoxin: HipB Mechanism: Unknown

Cold-Shock Response

In most environments free living bacterial cells are subjected to stress. This stress may be in the form of a change in pH, osmolarity, temperature, exposure to antibiotic compounds or nutritional starvation. Fluctuations in temperature have wide ranging effects on growth and survival and hence bacteria have developed mechanisms that allow them to adapt to these alterations. The evolution of the cold-shock response is one such mechanism. It helps exponentially growing cells cope with a downshift in temperature from their optimal growing temperature; typically a shift from 37°C to 15°C for *E. coli* [Thieringer *et al*., 1998]. After a sudden downshift in external temperature bacterial cells are presented with three problems: (1) decrease in membrane fluidity which influences cellular functions associated with the membrane; (2) transcriptional and translational impairment as a consequence of the stabilization of secondary structures of messenger ribonucleic acid (mRNA) and DNA respectively; (3) ribosomal malfunction [Phadtare *et al.*, 2004]. Directed synthesis of specific proteins, known as the family of cold-shock proteins (Csp), occurs during cold shock conditions. These include helicases, nucleases, transcription factors and nucleic-acid binding proteins (chaperones) [Weber et al., 2003, Inouye et al., 2004, Gualerzi et al., 2003]. Cold shock proteins are divided into two classes, based on their expression patterns [Thieringer et al., 1998, Yamanaka 1999]. Class I cold shock proteins are expressed at very low levels at physiological conditions (37°C); inducible greatly upon cold shock (15°C). CspA, CspB, CspG, CspI, CsdA, RbfA, NusA and PNP constitute this class. Class II proteins are expressed at average levels at 37°C and are moderately induced on cold shock. IF-2, H-NS, RecA, Trigger

Factor, pyruvate dehydrogenase and the α subunit of DNA gyrase are under Class II of cold shock proteins [Yamanaka 1999].

Physiologically, the response is divided into two phases: an initial and temporary arrest in growth, termed the acclimation phase, and a consecutive resumption in growth. The bulk of translation in inhibited in the acclimation phase and there is a decrease in saturation of fatty acids. As the cells adapt to the change in temperature, Csp synthesis decreases and normal growth is resumed [Jones et al., 1987].

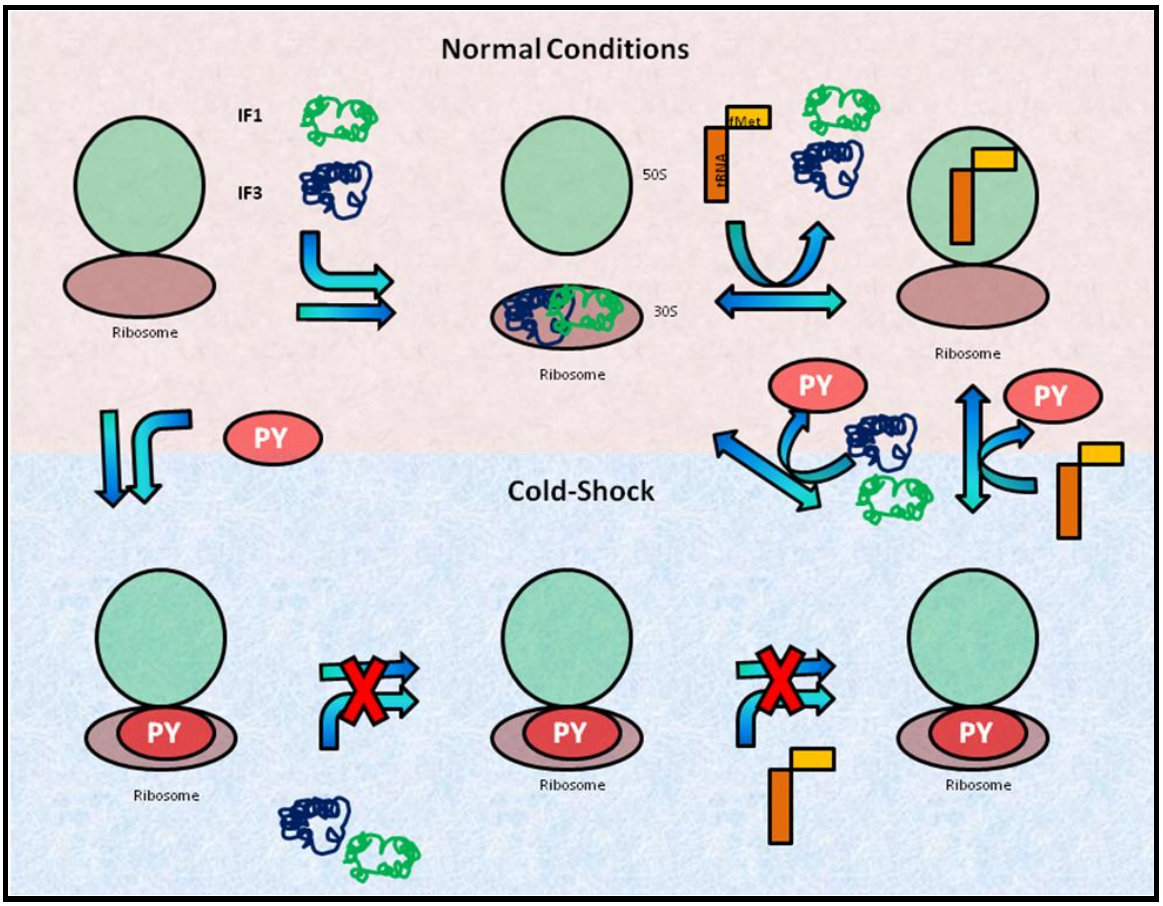
In contrast to heat shock conditions, no cold-specific σ (sigma) factor has been identified so far [Weber et al., 2003]. Many of the proteins associated with the cold-shock response in *E. coli* are linked with the translational apparatus: trigger factor, translation initiation factors IF1 and IF3, RNA chaperones and RNA helicase-like proteins (Graumann et al., 1998).

Protein Y

Organisms limit protein synthesis during environmental stress. The ribosome plays a central role in adapting to stress. It serves as a checkpoint for sensing temperature shifts [Vanbogelen et al., 1990; Cahsel et al., 1996] and nutrient levels [Ashe et al., 2000]. When stress is in the form of cold-shock a plethora of specific proteins are expressed and help the organism buffer itself against fluctuations in environmental temperature [Weber et al 2003, Inouye et al 2004]. Protein Y (PY) is one such protein associated with the cold-shock response. It has been identified as a product of the *yfiA* gene, an ORF located upstream of the *phe* operon of *E. coli* [Agafonov et al., 1999]. PY is also known as Ribosome associated inhibitor A (Rai-A), YfiA and spot Y. PY is a 12.7-kDa protein that associated with ribosomes upon the induction of cold-shock, as well as during stationary phase, stabilizing the monosomes against dissociation [Agafonov et al., 2001; Maki et al., 2000]. PY binds to the ribosome at the interface of the 50S and 30S subunits through specific interaction with the 30S subunit and prevents dissociation of the ribosome [Agafonov et al., 2001; Maki et al., 2000]. Initiation Factor 3 (IF3) is known to function in the opposite way; its binding to the 50S subunit prevents this subunit from associating with the 30S subunit [Subramanian et al., 1970]. During cold-shock PY blocks the peptidyl-tRNA site (P-site) and part of the aminoacyl-tRNA site (A site) of the ribosome [Vila-Sanjurjo et al., 2004]. This leads to inhibition of protein synthesis by blocking translation [Agafonov et al., 1999; Vila-Sanjurjo et al., 2004]. Furthermore PY competes with conserved translation initiation factors 1 (IF1) and 3 (IF3) [Vila-Sanjurjo et al., 2004]. In bacteria IF1 associates with the 30S ribosomal subunit in the A site and prevents an aminoacyl-tRNA from entering into the site. It also

modulates IF2 binding to the ribosome by increasing its affinity [Stringer et al., 1977]. IF3 is specially required in *E. coli* for the 30S subunit to bind to the initiation site in mRNA and also allows for rapid codon-anticodon pairing [Pon & Gualerzi, 1974]. When the environment returns to normal temperature (37°C) the affinity of PY for the ribosome diminishes and translation resumes.

Figure 2: Protein Y and the Cold Shock Response



(Adapted from Wilson and Nierhaus, 2004)

PY adopts a βαββββα topology and represents a compact two-layered sandwich of two α helices, that are nearly parallel, packed against the same side of a four-stranded β sheet. This structure is similar to that of dsRNA binding proteins. However PY does not seem to make direct contact with the 16S rRNA [Rak et al., 2002; Ye et al., 2002].

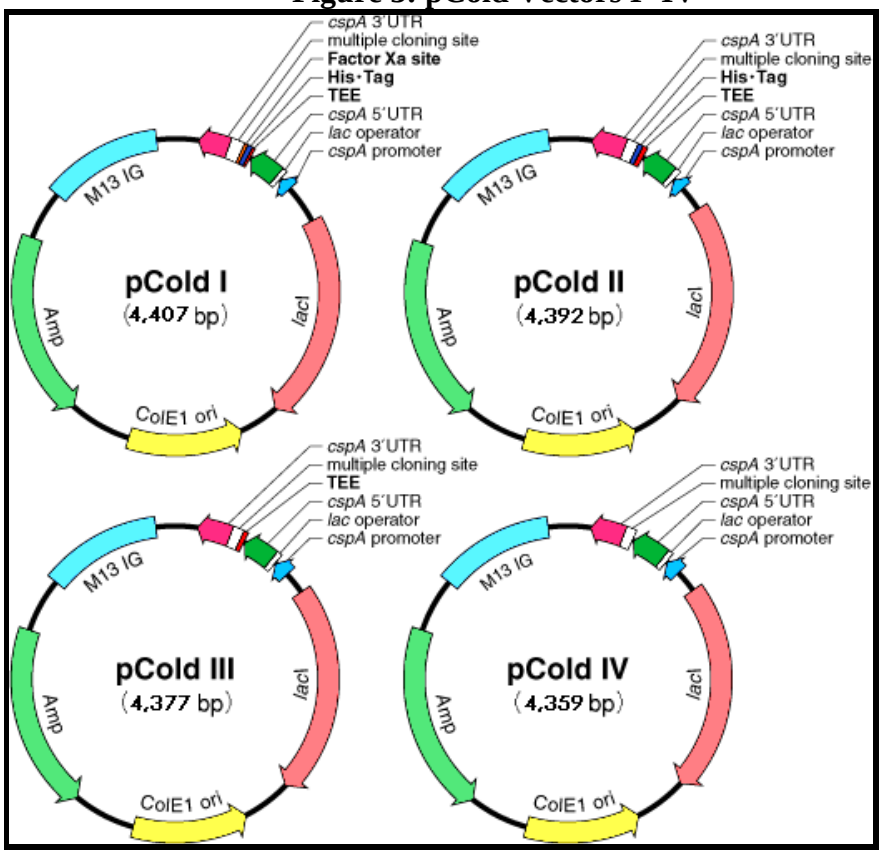
pCold Vectors

Expression at low temperature enhances protein stability and solubility [Qing et al., 2004]. pCold vectors (Takara Bio Inc, USA) have been shown to enable the expression of certain proteins that could not be expressed at high levels in the pET system [Qing et al., 2004]. Four pCold™ vectors have been developed for use at low temperatures (15°C) [Qing et al., 2004]. pCold™ DNA vectors I, II, III and IV (Takara Bio Inc,) are derived from the pUC18 vector that implement the *cspA* promoter, *cspA*5' and *cspA*3' UTR to facilitate high level expression at 15°C. CspA is the major *E. coli* cold-shock protein. Its activity is essential acclimation of *E. coli* to cold temperatures [Jiang et al., 1997; Phadatre & Inouye et al., 2004]. CspA acts as an RNA chaperone that unfolds secondary structures. Once an *E. coli* culture is transferred from normal growth temperature (37°C) to cold shock conditions (15°C), most mRNA translation is blocked. This occurs because the cells lack cold-inducible ribosomal factors that they need for formation of the translation initiation complex [Mitta et al., 1997; Phadtare et al., 2002]. Surprisingly, CspA mRNA does not need these factors and is readily produced at cold temperatures. Cold shock expression thereby allows selective induction of protein synthesis at 15°C, where protease activity is decreased and synthesis of cellular proteins is suppressed.

The four pCold™ vectors (Figure 3) developed differ from each other in the existence of the following: factor Xa cleavage sites, translation-enhancing element (TEE), and $(His)_6$ tags. All the vectors contain a *lac* operator that allows for controlled expression and multicloning sites downstream of the *cspA* promoter. pColdI, pColdIV

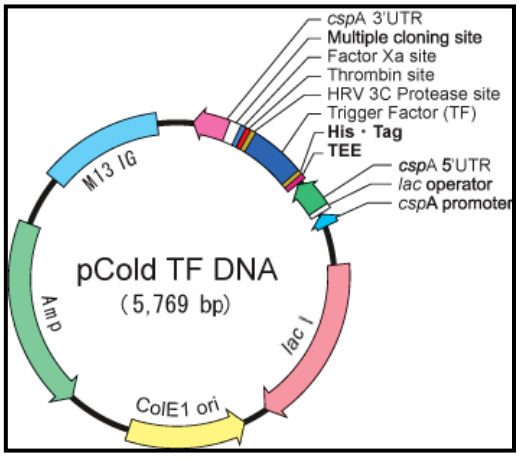
and pCold-TF were used in this work. pColdI and pCold-TF have a $(His)_6$ tag and these were utilize in protein purification experiments.

Figure 3: pCold Vectors I- IV



(Image from Takara Bio Inc, USA; www.takara-bio.com)

pCold-TF^{TM}: Takara's pCold –TF vector (Figure 4) expresses the ribosome-associated chaperone Trigger Factor (TF) as a soluble tag. TF in this *E. coli* fusion cold shock expression system facilitates co-translational folding of newly expressed proteins, thereby increasing expression of soluble, active protein. pCold-TF^{TM} also has a $(His)_6$ tag as shown.



(Image from Takara Bio Inc, USA; www.takara-bio.com)

Figure 4: pCold-TF^{TM} vector

b2596 and *yfiA*

PY stabilizes the monosomes against dissociation into 50S and 30S subparticles, leading to translation inhibition. This mechanism of action of *yfiA* resembles that of a TA system toxin and made us search for its cognate antitoxin. We found a small hypothetical ORF, designated *b2596*, upstream of *yfiA*, and this is the target of my project. Sequence analyses of the two genes indicated that they have homology with bacterial TA systems, which have been implicated in various stress responses. The two genes encode small proteins and have opposing pIs. B2596 is 69 amino acids in length and PY is 203 amino acids long. The predicted pI for B2596 is 11.3, which is in the basic range, and that for PY is 6.57, in the acidic range. These traits are characteristic to TA modules and encouraged us to study *b2596* and *yfiA* as a novel TA system.

We propose the name 'Protein X' (PX) for the product of gene *b2596*. In this work 'PX' has been used to represent the protein encoded by *b2596.*

Materials and Methods

Strains, Plasmids and Reagents

The *E. coli* strains BL21(DE3)(F-*omp*T *hsd*S_β(r_β-m_β-) *dcm gal*(DE3) tonA) (Novagen Inc., USA) and CD43(DE3) (F-*omp*T *gal hsd*SB (r_β-m_β-) *dcm lon* λDE3) [Miroux and Walker, 1996], a strain derived from BL21(DE3) were used for all protein expression studies, unless noted otherwise. CD43(DE3) cells are double mutants from BL21(DE3) cells in which both subunits b and c of the F-ATPase, an alanine-H^+symporter, and the ADP/ATP and the phosphate carriers from mitochondria are over-produced [Miroux and Walker, 1996]. *Taq* polymerase was used for all Polymerase Chain Reactions (PCR) and the oligonucleotides used were synthesized by IDT (Coralville, IA). For restriction digests plasmid DNA was obtained using a standard Alkaline Lysis Miniprep Protocol (Sambrook et al., 2001). *E. coli* K-12 Mach-T1(Δ*rec*A 1398 *end*A1 *ton*A Φ80Δ*lac*M15 Δ*lac*X74 *hsd*R (r_k^--m_k^+-) ((Invitrogen, Carlsbad, CA) were used for all cloning experiments. The sequence of all plasmids with PCR inserts was confirmed by automated DNA sequence analysis (Genewiz Inc., NJ, USA) of DNA prepared by Qiaprep Miniprep Kit (QiaGen, Valencia, CA). QiaGen Kits were also used for gel purifications and PCR as necessary during the preparation of recombinant plasmids. BW25113 (*lac*I^qnB_{T14}*Δlac*Z_{WJ16} *hsd*r514 *Δara BAD*$_{AH33}$*Δrha BAD*$_{LD78}$) (Datsenko and Wanner, 2000), genomic DNA and whole cells were used as templates to clone out *b2596* and *yfiA*. BW25113 genomic DNA was also used to make some probes used in Northern analyses. BW25113 cells were also used for all RNA extractions using Hot-Phenol RNA Extraction Protocol (Verwoerd et al., 1989). All enzymes were from New England BioLabs (Ipswich, MA). Unless otherwise noted, all bacterial cultures were

grown in Luria Broth (LB) media. Ampicillin (Amp) and Kanamycin (Kan) were used as needed at concentrations of 100 µl/mL and 33µ/mL.

For a list of all cloning primers used please refer to Table 4. For a list of all plasmids used please refer to Table 5.

Table 4: Primer List

Name	Sequence
NWO1176	5'-gctgaattcaccaagacgggaagacaagagg-3'
NWO964	5'-cgggatccctcttcttcaacttcttcgacgaag-3'
NWO963	5'-ggaattccatatgagctgaagtttttttattctgtcagttg-3'
NWO962	5'-cgggatcctcagcataccgttcctgatgtcaaaatg -3'
NWO961	5'-ggaattccatatgttgagctgccgtttttttattctgtcagttg-3'
NWO960	5'-cggcatccctactcttcttcaacttcttcgacgaag-3'
NWO959	5'-ggaattccatatgatgacaatgaacattaccagcaaacaaatg-3'
NWO958	5'-2atttgatgagaatcgatagcga-3'
NWO1228	5'-cgccaacgcgccttcgggcgcg-3'
NWO1229	5'-gtacagtacccgtactgttttcacgctgtc-3'
NWO956	5'-gacggtctgcgacatgttggcggat-3'
NWO1120	5'-ccgttcctgatgtcaaaatgtgtgatgaa-3'
NWO1119	5'-cgtctcgccaaactggaaaaatggcaaacacatc-3'
NWO1118	5'-cctaacggcgttctggttgccagtggtaaacat-3'

Clone Construction (Work Contributed in part, by Meredith Prysak & Jennifer Hurley**):**

b2596 was PCR amplified from *E. coli* genomic DNA using primers NWO961 and NOW962, and cloned into pColdI's *Nde*I/*Bam*HI restrictions sites. This places a $(His)_6$ tag in-frame at the N-terminus. *b2596* was also cloned into pColdIV, no $(His)_6$ tag, in a similar manner. *b2596* was also PCR amplified from genomic DNA with primers NWO963 and NWO964, mutagenizing the natural TTG start codon to an ATG, and cloned into the *Nde*I/*Bam*HI sites of pColdI (designated *b2596(ATG)*-pColdI). This modified *b2596* was also cloned into the *Nde*I/*Bam*HI of pET28a, creating an N-terminus $(His)_6$ tag on *b2596*. *yfiA* was PCR amplified from genomic DNA with primers

NWO959 and NWO960, and cloned into the *Nde*I/*Bam*HI sites of pColdIV. It was also cloned into the *NdeI*/*Xho*I sites of pET21c, placing a C-terminal $(His)_6$ tag on *yfiA*. The *b2596-yfiA* module was PCR amplified from genomic DNA with primers NWO961 and NWO960, and cloned into the *Nde*I/*Bam*HI sites of pET28a, creating a N-terminal $(His)_6$ tag on *b2596*. The clones described so far were constructed by Meredith Prysak and Jennifer Hurley.

Table 5: Plasmids

Name	Description
***b2596(ATG)*-pColdI**[1]	*b2596(ATG)* in pColdI, $(His)_6$ tagged, *Nde*I/*Bam*HI sites, inducible (IPTG)
***b2596(ATG)* -pET28a**[1]	*b2596(ATG)* in pET28a, N-terminal $(His)_6$-tagged, *Nde*I/*Bam*HI sites, inducible (IPTG)
***b2596* -pET28a**	*b2596*in pET28a, N-terminal $(His)_6$-tagged, *Nde*I/*Bam*HI sites, inducible (IPTG)
***b2596*-pColdI**[1]	*b2596* in pColdI, $(His)_6$ tagged, *Nde*I/*Bam*HI sites, inducible (IPTG)
***b2596/yfiA*-pET28a**[1]	*b2596-yfiA* in pET28a, N- terminal $(His)_6$-tagged, *Nde*I/*Bam*HI sites, inducible (IPTG)
***b2596(ATG)/yfiA*-pET28a**[1]	*b2596(ATG)-yfiA* in pET28a, N- terminal $(His)_6$-tagged, *Nde*I/*Bam*HI sites, inducible (IPTG)
***YfiA*-pColdIV**[1]	*yfiA* in pColdIV, *Nde*I/*Bam*HI sites, inducible (IPTG)
b2596-pColdIV[1]	*b2596* in pColdIV, *Nde*I/*Bam*HI sites, inducible (IPTG)
***yfiA*-pET21c**[1]	*yfiA* in pET21c, C-terminal $(His)_6$-tagged, *Nde*I/*Xho*I sites, IPTG inducible
***b2596*-pCold-TF**	*b2596* in pCold-TF, $(His)_6$-tagged, TF-tagged, *Nde*I/*Bam*HI sites, inducible (IPTG)

[1]These clones were made by Meredith Prysak and Jennifer Hurley

b2596 was PCR amplified from *E. coli* genomic DNA using primers NWO961 and NOW962, and cloned into the *Nde*I/*Bam*HI restrictions sites pCold-Trigger Factor (pCold-TF)(Takara Bio Inc, USA). Trigger factor had a 3' $(His)_6$ tag in this construct.

b2596 mRNA Expression Analysis by RT-PCR

To test for the presence and levels of *b2596* mRNA we performed an Reverse Transcriptase Polymerase Chain Reaction, (RT)-PCR, on RNA extracted from induced (1 mM IPTG) and uninduced BW25113 cells harboring *b2596*(ATG)-pColdI. A 50mL culture was grown up to an OD_{600} of 0.3-0.35 at 37°C. The culture was then acclimated at 15°C for 30 minutes and split into two. One culture was induced, and the other left uninduced, for 24 hours. Total RNA was extracted using the hot phenol method, as previously described [Sarmientos, 1983], from each culture and reverse transcriptase reactions were carried out on equal amounts of total RNA. AMV-RT buffer and AMV-RT enzyme (New England Biolabs, Ipswich, MA) were used for the reaction. The primer used was NWO961. Negative control samples, containing no AMV-RT enzyme were also prepared for both the uninduced and induced RNA samples. The reverse transcriptase was then heat inactivated for 10 minutes at 75°C, and all samples were treated with DNase-free RNase for 10 minutes. These reactions were then used as template for the PCR reaction using primers NWO961 and NWO962. PCR reactions were then run on 0.8% agarose gels and stained with ethidium bromide for visualization.

Transcriptional Start Site Analyses of *b2596* and *yfiA* (Work Contributed by Jennifer Hurley)**:**

For *in vivo* primer extension analysis of mRNA cleavage sites *E. coli* BW25113 cells were grown to an OD of 0.2. The culture was then split into two and one half was allowed to acclimate for 30 minutes at 15°C and the other remained at 37°C. Total RNA was extracted using the hot phenol method as previously described [Sarmientos, 1983 #67] from each culture. Primer extension was carried out using primers which were 5'-labeled with [{γ}-^{32}P] ATP using T4 polynucleotide kinase. NWO958 was used for *b2596* and NWO956 was used for *yfiA* (Table 4). The labeled primer was incubated with 30 µl of RNA in reverse transcriptase buffer (NEB) at 65°C for 2 minutes and allowed to cool to room temperature for 30 minutes to bind the primer to the RNA. The primer/RNA complex was then incubated with RNase Inhibitor (20 units-NEB), reverse transcriptase (5 units-NEB) and 1mM final concentration of each dNTP. Primer extension was carried out at 42 °C for 1 h. The reactions were stopped by adding the 12 µl of sequence loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol EF). The DNA sequencing ladder used to analyze the cleavage recognition sequence was prepared using Sequenase Version 2.0 DNA Sequencing Kit (USB). *yfiA*-TOPO and *b2596*-TOPO were used as templates for the reaction, along with the same primers from the primer extension reactions. The sequencing ladder and the primer extension products were heated to 95 °C for 5 minutes and then cooled on ice for 3 minutes. They were loaded on by a 6% polyacrylamide/8 M urea sequencing gel and run at 1600V and exposed to film.

Protein Expression and Purification of *b2596* and *yfiA* (PY)

Table 6 describes the different *b2596* expression experiments that were carried out in different host strains using the vectors listed in Table 6. *b2596*-pColdI, *b2596*(ATG)-pColdI, *b2596*(ATG)/*yfiA*-pET28a or *b2596*-pET28a were transformed into BL-21(DE3) cells (listed in the table as BL-21) and C43(DE3) cells to evaluate expression of *b2596*. Cultures were grown in LB media at 37°C until the OD_{600} reached 0.2-0.25 for pET vector cultures and 0.3-0.4 for pCold vector cultures. All cultures were now split into two halves. One part of cultures in which the pET system was being used for expression was induced right away with 1 mM IPTG, the other half was used as the uninduced control. Cultures in which the pCold vector system was being used were shifted to 15°C and allowed to acclimate for 30 minutes. One half of these were then induced with 1 mM IPTG. Cells were harvested from all cultures just before and after induction. In addition, for all pET vector containing cultures cells were harvested at 1, 2, 3, 4 and 6 hours post induction and for all pCold vector containing cultures cells were harvested at 2, 4, 6 and 24 hours post induction. This is referred to as 'Time Course' in Table 6. Total protein was extracted from all and the samples were run on 17.5% SDS-PAGE gels and stained with Coomassie Blue for analysis. Where indicated in Table 6, the SDS-PAGE gels were analyzed by Western Blot Analysis using the Universal His Western Blot Kit 2.0 (Clontech Laboratories Inc, CA) to test for expression. For some sets of cultures, at 24 hour (for pCold systems) and the 6 hour (for pET systems) the cells were lyzed using a French Press and the protein extracts were applied to a Ni-NTA resin (Qiagen Protein Expression Kit) in a column. The columns were washed as per manufacturer's suggestions with a wash buffer containing 20 mM imidazole and then an

elution buffer containing 250 mM imidazole was used to get the purified protein off the Ni-NTA column. Elution fractions were then run on a 17.5% SDS-PAGE gel and stained with Coomassie Blue to visualize protein content.

b2596-pColdI, *b2596(*ATG)-pColdI or *b2596*-pET28a were also transformed into 'Magic' cells (obtained from the Cheryl Arrowsmith laboratory, University of Toronto, Canada) and expression was tested in LB and M9-minimal media separately. Magic cells are designed to help expression of proteins that may contain rare codons in their mRNA sequence. These cells contain genes that express the corresponding amino-acyl tRNA of the amino acids that are encoded by rare codons in the test-gene.

b2596-pCold-TF was transformed into BL-21(DE3) cells. A culture was grown in LB at 37°C until the OD_{600} reached 0.3-0.4, and shifted to 15°C and allowed to acclimate for 30 minutes. This culture was then split into two and one half was induced with 1 mM IPTG and 1ml of cells were harvested just before and after induction and at 1, 2, 3, 4, 6 and 24 hours post-induction and total protein was extracted. The samples were then run on a 17.5% SDS-PAGE gel and stained with Coomassie Blue for visualization. The 4, 6 and 24 hour post induction samples were applied to as Ni-NTA column as described above and the elution fractions obtained were run on an SDS-PAGE gel and stained with Coomassie Blue.

yfiA-pET21c was transformed into BL-21(DE3) cells. A 400ml culture was grown in LB at 37°C until an $O.D_{600}$ in the range 0.2-0.3 was observed. The culture was then allowed to acclimate at 15°C and induced with 1 mM IPTG. The culture was grown for 24 hours post-induction. The cells were sonicated to split them and protein (PY) was extracted from the lyzed cells. This protein sample was then used to test interaction

between B2596 and PY, $(His)_6$ tagged,as described in the next section. *yfiA*-pColdIV was transformed into BL-21(DE3) cells and this was also expressed and PY was extracted in a similar manner. Interaction was tested between this PY product, which had no $(His)_6$ tag, and TF-B2596 as described below.

Table 6: Protein X Expression Experiments: A list of all vectors, conditions and techniques used for testing expression of PX.

Strain	Vector	Expression	Media	Temp.	O.D induced at	*b2596* start site	Culture volume	Techniques
BL-21	pColdI	-	LB	15°C	0.2360	ATG	50mL	Time course, SDS-PAGE Gel
	pColdI	-	LB	15°C	0.2706	TTG	50mL	
	pET28a[1]	-	LB	37°C	0.2370	ATG	50mL	
	pET28a	-	LB	37°C	0.2473	TTG	50mL	
BL-21	pColdI	-	LB	15°C	0.2111	ATG	50mL	Time Course, Ni-column, SDS-PAGE Gels
	pColdI	-	LB	15°C	0.1770	TTG	50mL	
	pET28a[1]	-	LB	37°C	0.1700	ATG	50mL	
	pET28a	-	LB	37°C	0.2901	TTG	50mL	
C43(DE3)	pColdI	-	LB	15°C	0.2505	ATG	500mL	Time Course, Ni-column, Western Blot[2], SDS-PAGE Gels
	pColdI	-	LB	15°C	0.3508	TTG	500mL	
	pET28a[1]	-	LB	37°C	0.1952	ATG	500mL	
	pET28a	-	LB	37°C	0.2173	TTG	500mL	
CD43(DE3)	pET28a[1]	-	LB	37°C	0.3261	ATG	500mL	Time Course, Ni-column, Elution Test[3], Western Blot[2], SDS-PAGE Gels
	pET28a	-	LB	37°C	0.3327	TTG	500mL	
Magic[d]	pColdI	-	LB	15°C	0.3391	ATG	250mL	Time Course, SDS-PAGE Gel
	pColdI	-	LB	15°C	0.3433	TTG	250mL	
	pET28a	-	LB	37°C	0.1952	ATG	250mL	
Magic[d]	pColdI	-	M9	15°C	0.3897	ATG	50mL	Time Course, SDS-PAGE Gel
	pColdI	-	M9	15°C	0.3317	TTG	50mL	
	pET28a	-	M9	37°C	0.2250	ATG	50mL	
BL-21	pCold-TF	+	LB	15°C	0.3803	TTG	500mL	Time Course, Ni-column, SDS-PAGE Gel

[a] The vector contained the both, *yfiA* and *b2596*

[b] Universal His Western Blot Kit 2.0, Clontech Laboratories Inc, CA

[c] Eluted with increasing imidazole concentrations

[d] Strain from Dr. Cheryl Arrowsmith, University of Toronto, Canada

Northern Blot Analyses

Total cellular bacterial RNA was prepared by the hot phenol method (Sarmientos et al., 1983) from *E. coli* BW25113 cells that had been grown to an OD of 0.2. Total RNA was then resuspended in diethylpyrocarbonate (DEPC)-treated water to a final concentration of 1.5-4.5 µg/uL, 1X MOPS, 17.5% formaldehyde, 50% deionized formamide, and ethidium bromide (0.5 uL). The samples were heated at 65°C for 15 minutes and then mixed with 2 µL of RNA Loading Buffer (50% formamide, 1X MOPS, 18.5% formaldehyde, 0.5-0.7% glycerol, bromophenol blue powder for color). Electrophoresis was performed in a fume hood at 120V on a 1.2% agarose, 10X MOPS and 5.4% formaldehyde gel. All reagents were prepared with DEPC-treated water.

The samples were then visualized by ultraviolet (UV) lamp and the RNA ladder was marked on the gel. Next the gel was rocked in 0.05 N NaOH for 20 minutes, followed by a 40 minute soak in 20X SSC (3.0 M NaCl, 0.3 M Sodium citrate). The samples were then transferred by diffusion onto a nitrocellulose membrane in 10X SSC overnight and subsequently washed with 2X SSC. The RNA samples were crosslinked to the nitrocellulose membrane by baking the membrane at 80°C for 2 hours.

The membrane was then placed in a hybridization bottle containing 5 mL of hybridization mixture [50% formamide, 5X SSPE (3.0M Sodium Chloride, 0.2M Sodium Hydrogen Phosphate, 0.02 M EDTA, pH 7.4), 1X Denhardt's Solution, 0.3% SDS, 0.1 mg of ssDNA/mL] and rotated in a hybridization oven for two hours at 37°C to pre-hybridize the membrane. Then, 200 µL of the hybridization mix was removed from the bottle and added with α-^{32}P-labelled oligonucleotide in a separate tube, and denatured for five minutes at 95°C for random primer labeling (Roche Diagnostics). Three different set

of DNA fragments were used to hybridize to the membranes in three separate northern blot analyses. The primers used to generate these DNA fragments have been described at the end of this section. The hybridization mixture containing the probe was added back into the bottle containing the membrane and allowed to hybridize overnight at 37°C.

The membrane was washed two times for 15 minutes at 37°C and then at 42°C, a total of four washes, in 2X SSPE, 0.2% SDS wash buffer. The hybridized filter was then exposed to film for different time periods and the film was analyzed by autoradiography.

Table 7: Primers for Northern Analyses

Probe	Aim
NWO1176	To test for RNA in immediate 5' region of *yfiA*
b2596	To test for *b2596* RNA, used *yfiA* as a positive control
b2596 fragment	To test for *b2596* RNA using a partial-*b2596* fragment (made through PCR using primer NWO961 and NWO958)

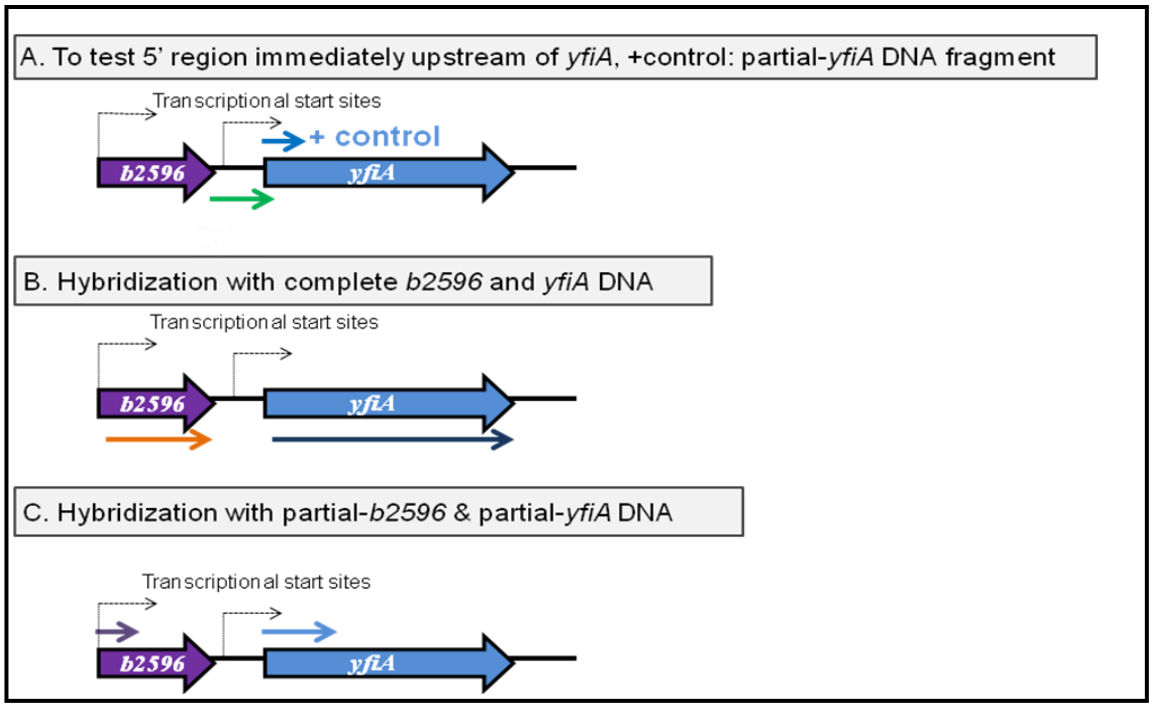


Figure 5: DNA fragments used in Northern Analyses: Arrows indicate position of hybridization fragments designed for Northern analyses.

Protein X and Protein Y Interaction Studies

To test whether the product of gene *b2596*, Protein X, interacts with Protein Y, the product of the *yfiA* gene, both proteins were purified and interaction was tested using Ni-NTA columns, described as follows.

b2596 was expressed as a fusion protein with trigger factor in pCold-TF. Cells from this expression experiment were lyzed using sonication and protein was extracted from the lyzed cells. 15 mL of this extract was mixed with 15 mL of PY extract (expressed in pColdIV) and 1 mL of Ni-NTA resin. This mix was rocked for 1 hour at 4°C and then poured over a Ni-NTA column. The columns were washed as per manufacturer's suggestions with a wash buffer containing 20mM imidazole and then an elution buffer containing 250 mM imidazole was used to get the bound protein off the Ni-NTA column. Elution fractions were then run on a 17.5% SDS-PAGE gel and stained with Coomassie Blue to visualize protein content.

In another test for interaction, PX was cleaved off of the PX-TF fusion protein using a Thrombin Kit and Thrombin Cleavage Protocol (Novagen, Inc., USA). The PX and trigger factor mix was then applied to a Ni-column to obtain pure PX. Trigger factor has a $(His)_6$ tag in this pair. 10mL of flow through from the column, containing relatively pure B2596, was then mixed with 10mL of PY extract and 1mL of Ni-NTA resin and rocked for 1 hour at 4°C to enable allow for interaction. The mix was then applied to a Ni-NTA column and elution fractions were obtained as described earlier. These elution fractions were run on a 17.5% SDS-PAGE gel and silver-stained to visualize protein content.

Protein X and Protein Y Growth Profiles

All liquid culture toxicity assays for *E. coli* cells were conducted in LB media with fresh transformants as follows: a 100 mL culture of BL-21(DE3) cells harboring *b2596*-pColdIV or *yfiA*-pColdIV was grown to exponential phase (OD_{600} =0.3-0.4 or OD_{600} =0.2-0.3) at 37°C, and the culture was then split into two 50 mL cultures. The cultures were then shifted to 15°C and allowed to acclimate for 30 minutes. One of each set of cultures was induced with 1mM IPTG and an equivalent volume of sterile H_2O was added to the other (uninduced). At and after induction 1 ml of culture was removed from each flask hourly for 6 hours and then at the time points indicated until 24 hours to measure the OD_{600}. The results were plotted on a linear graph.

Results

PY inhibits translation, mainly at the elongation phase, by blocking the binding of aminoacyl-tRNA to the ribosomal A site [Agafonov et al., 2001]. This mechanism of action of *yfiA* resembles some TA system toxins, and directed us to examine whether PY had a cognate antitoxin.

We found a small hypothetical ORF, designated *b2596*, upstream of *yfiA*. We started to look for parallels that could be drawn between the canonical proteic TA module (Figure 1) and the *b2596-yfiA* module. TA systems play a role in bacterial stress response and since PY helps bacterial cells cope with cold-shock stress we wanted to explore the possibility that *b2596-yfiA* is a TA module that is triggered by cold-shock. We found that the *b2596-yfiA* module abided by a significant number of the canonical TA module characteristics (Table 8). The two genes are adjacent to each other, with the antitoxin preceding the toxin. Both *yfiA* and *b2596* encode small proteins; PY is 114 amino acids with a molecular weight of 12.7 kDa and PX is 69 amino acids with a predicted molecular weight of 7.8 kDa. These proteins have opposing charges; PX is basic with a pI of 11.3 and PY is acidic with a pI of 6.57. This trend matches that seen in other TA systems; the antitoxin is usually basic and the toxin usually acidic. The genes show sequence similarity to known TA modules. PX shows a 24.3% similarity to Phd and 19.3% to DinJ. PY shows a 21.4% sequence similarity to Doc (Phd's cognate toxin) and 27% to YafQ (DinJ's cognate toxin) (Figure 6, 7). Thus, our initial investigation demonstrated that *b2596-yfiA* exhibits properties characteristic to TA modules. To examine *b2596* and *yfiA* further, we performed more detailed experiments as discussed in later sections.

Table 8: Comparison of *b2596-yfiA* to Canonical TA module Features: Features listed in blue boxes match perfectly. Feature(s) listed in green are permissible for TA systems since they conform to those seen in some TA modules that are exceptions to the norm.

	Characteristics of TA Modules	**Characteristics of *b2596* and *yfiA***
Sequence similarity	Exhibit sequence similarity among family members	**B2596*:*** 24.3% sequence similarity to **Phd,** 19.3% to **DinJ** **PY :** 21.4% sequence similarity to **Doc**, 27% to **YafQ**
pI values	Toxin and antitoxin have pI values that lie in the acidic and basic range respectively	***b2596*** :11.3 ***yfiA***: 6.57
Protein size	Toxin and antitoxin proteins are relatively small	***b2596*** : 69 amino acids **PY**: 114 amino acids, PX: 7.8kD (predicted) PY:12.7kD
Toxicity	Over expression of free toxin is toxic to the cell and causes growth arrest	PY binds to the 30S ribosomal subunit in cold-shock conditions, causes growth inhibition
Autoregulation	TA complex binds to an upstream palindrome and autoregulates the TA operon	28 base pair palindrome suggested upstream /within *b2596*, possibly regulated by cold-shock originally
Gene positions	The two genes are adjacent; the antitoxin preceding the toxin	*b2596* precedes *yfiA*
Intergenic distance	ORFs of the two genes overlap by a few base pairs or have a 1-2 base distance in between	34 base pair distance between *b2596* and *yfiA*
Promoter	Toxin and antitoxin genes have a common promoter	Transcribed separately
TA Complex	Antitoxin and toxin form a stable complex, in stress-free conditions	Unknown
Antitoxin	Antitoxin is usually labile as compared to the toxin	Unknown

Figure 6: Alignment Data for Protein X and Phd, and Protein Y and Doc

Alignment of Sequencing Data for PX and Phd: 24.3% Sequence Similarity, 13.5% Identity

```
Phd, Bacteriophage P1:(1)  MQSINFRTARGNLSEV-LNNVEAGEEVEITRRGREPAVIVSKATFEAYKKAALDAEFASL   (59)
                                        LS V L         +     R  +   +     ++KK +L      +
PX, E. coli:           (1)  -----LSCRFFILSVVKLKRFSRYRSHQIWLALRYSSSKKTSLPAISHKKDSLTKSDKIM   (55)

Phd, Bacteriophage P1:(60) FDTLDSTNKELVNR                                              (73)
                             +        V
PX, E. coli:           (56) RFSSHILTSGTV-C                                             (68)
```

Alignment of Sequencing Data for PY and Doc: 21.4% Sequence similarity, 11.1% Identity

```
Doc, Bacteriophage P1:(1)  MRHISPEELIALHDANISRYGGLPGMSDPGRAEAIIGRVQARVAYEEITDLFEVSATYLV   (60)
                           M      + + +  A            +  +   I          +       ++    V
PY,E. coli:            (1)  MTMNITSKQMEITPAIRQHVADRLAKLEKWQTHLINPHIILSKEPQGFVADATINTPNGV   (60)

Doc, Bacteriophage P1:(61) ATARGHIFNDANKRTALNSALLFLRRNGVQVFDSPELADLTVGAATGEISVSSVADTLRR   (120)
                           A G   +         L + L     N +Q       A  +V  A     V
PY,E. coli:            (61) LVASGKHEDMYTAINELINKLERQ-LNKLQHKGEARRAATSVKDANFVEEVEEE------   (114)
```

Figure 7: Alignment Data for Protein X and DinJ, and Protein Y and YafQ

Alignment of Sequencing Data for PX and DinJ: 19.3% Sequence Similarity, 12.5% Identity

```
DinJ, E. coli:        (1)  MAANAFVRARIDEDLKNQAADVLAGMGLTISDLVR-ITLTKVAREKALPFDLREPNQL-T   (58)
                                                L++  L R           AL +   +   l
PX, E. coli:          (1)  --------------------LSCRFFILSVVKLKRFSRYRSHQIWLALRYSSSKKTSLPA   (40)

DinJ, E. coli:        (59) IQSIKNSEAGIDVHKAKDADDLFDKLGI                                  (86)
                           I   K+S    D      +  L
PX, E. coli:          (41) ISHKKDSLTKSDKIMRFSSHILTSGTV-C                                 (68)
```

Alignment of Sequencing Data for PY and YafQ: 27% Sequence similarity, 19.1% Identity

```
YafQ, E. coli:        (1)  MIQRDIEYSGQYSKDVKLAQKRHKDMNKLKYKMTLLINNTLPL---PAVYKDHPLQGSWK   (59)
                           M    I  S        + Q     + KL+   T LIN  + L   P  +       +
PY,E. coli:           (1)  MTMN-IT-SKQMEITPAIRQHVADRLAKLEKWQTHLINPHIILSKEPQGFVADATINTPN   (59)

YafQ, E. coli:        (60) GYRDAHVEP-DWIL-IYKLTDKLLRFERTGTHAALFG*-----------------        (95)
                           G   A  +  D    I +L +KL R      H
PY,E. coli:           (60) GVLVASGKHEDMYTAINELINKLERQLNKLQHKGEARRAATSVKDANFVEEVEEE*       (114)
```

Sequence Analysis of *b2596-yfiA* DNA and its upstream region

We analyzed the DNA sequence upstream of and around the translational start site of *b2596* and the intergenic region between *b2596* and *yfiA* to check for regulatory sequences because all known TA modules are autoregulated by both antitoxin alone and the TA complex. We found a 28 base pair palindrome that spanned the TTG start site of *b2596.* This palindrome had a one base mismatch. A putative -10 region and -35 region showing matches to the consensus sequence for the σ 70 promoter was also seen (Figure 8A).

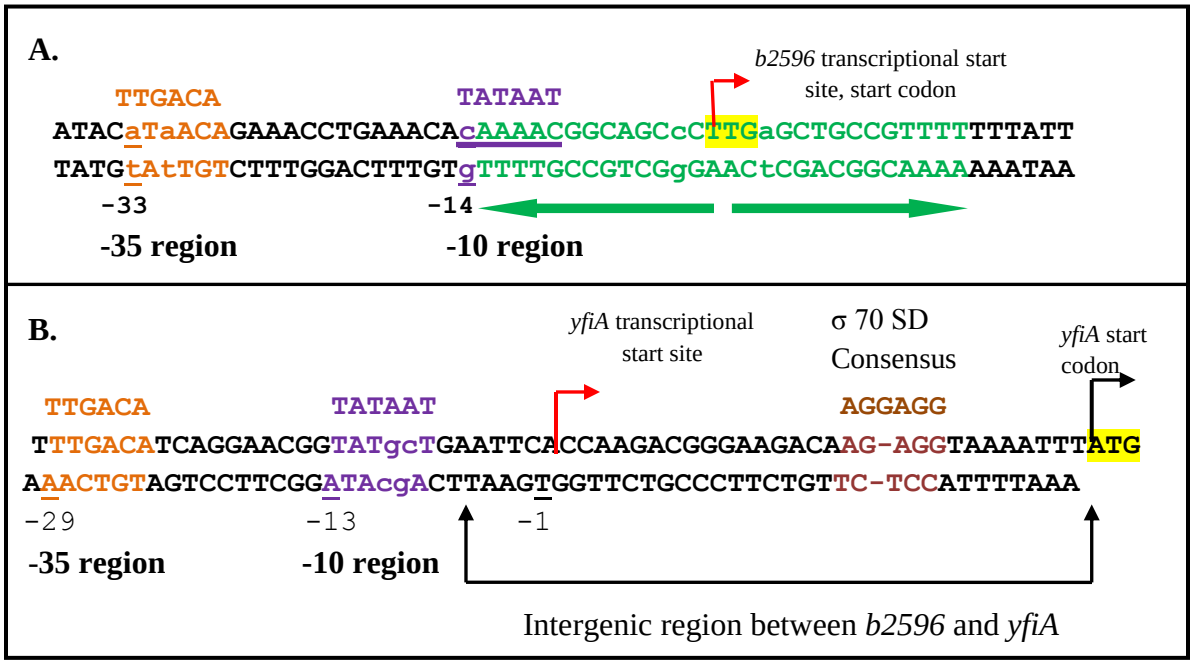


Figure 8: Sequence analysis of *b2596-yfiA* DNA and its upstream region. The red arrow in A and B indicates the predicted transcriptional start sites, start codons for both genes have been highlighted in yellow. **A**. Palindrome identified spanning the *b2596* start codon, shown in **green**. Putative -10 region is shown in **purple** and **green,** -35 region in **orange**. **B.** Putative -10 and -35 regions are in **purple** and **orange** respectively and show consensus for σ70 promoter. A sequence matching SD consensus was also identified; it is missing one base (G).

We found a putative -10 and -35 region showing matches to the consensus sequence of the σ 70 promoter upstream of the predicted *yfiA* transcriptional start site

(Figure 8B). A sequence matching, albeit missing one base (G), the Shine Dalgarno (SD) consensus sequence (AGGAGG) was also seen upstream of the putative -10 region, upstream of *yfiA.* We also identified a 26 base pair palindrome approximately 200 bases upstream of *yfiA*, within the sequence of *b2596*. However, this palindrome does not appear to be a significant in possible regulation of *yfiA* due to its distance from the gene.

b2596 mRNA Expression Analysis by RT-PCR

To check for *b2596* mRNA expression RT-PCR was performed on whole cell RNA extracted from BW25113 cells harboring *b2596*(ATG)-pColdI. The results showed that we could indeed induce *b2596* mRNA expression. All calculations were normalized to uninduced negative control. The induced sample showed a 10-fold increase in *b2596* mRNA levels (Figure 9).

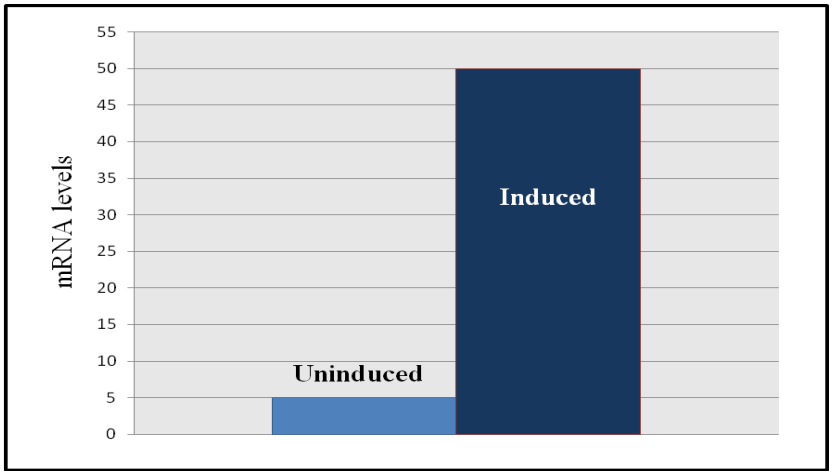


Figure 9: *b2596* mRNA Expression Analysis. RT-PCR results were quantified from the bands (intensity of ethidium bromide UV excitation) seen on the 0.8% agarose gel and all calculations were normalized to the uninduced negative control. The induced *b2596*(ATG)-pColdI, the **navy blue** bar, sample showed mRNA levels 10 folds higher than those seen in the uninduced sample.

Transcriptional Start Site Analyses of *b2596* and *yfiA*

All known TA gene pairs are in an operon, under the control of a single promoter. As another step towards characterizing the *b2596-yfiA* module in this regard we sought to identify the transcriptional start sites for both genes. We performed primer extension analyses on RNA extracted from *E. coli* BW25113 cells using primers near the 3' end of their respective genes (Figure 10). We observed independent transcriptional start sites for both genes. The *yfiA* transcript started 29 bases upstream of the AUG codon, in the intergenic region between *yfiA* and *b2596*. For *b2596*, transcription began at the UUG start site, indicating that *b2596* encodes a leaderless mRNA transcript. Therefore, we did not observe evidence that the *b2596-yfiA* genes are cotranscribed in a bicistronic message.

Figure 10: Transcriptional Start Site Analyses of *b2596* and *yfiA*
A. Start sites for *b2596* and *yfiA* indicated on the DNA sequence. The start site for *b2596* is shown with a blue arrow immediately at the TTG start site. The start site for yfiA is shown by a red arrow in the intergenic region between *yfiA* and *b2596*.

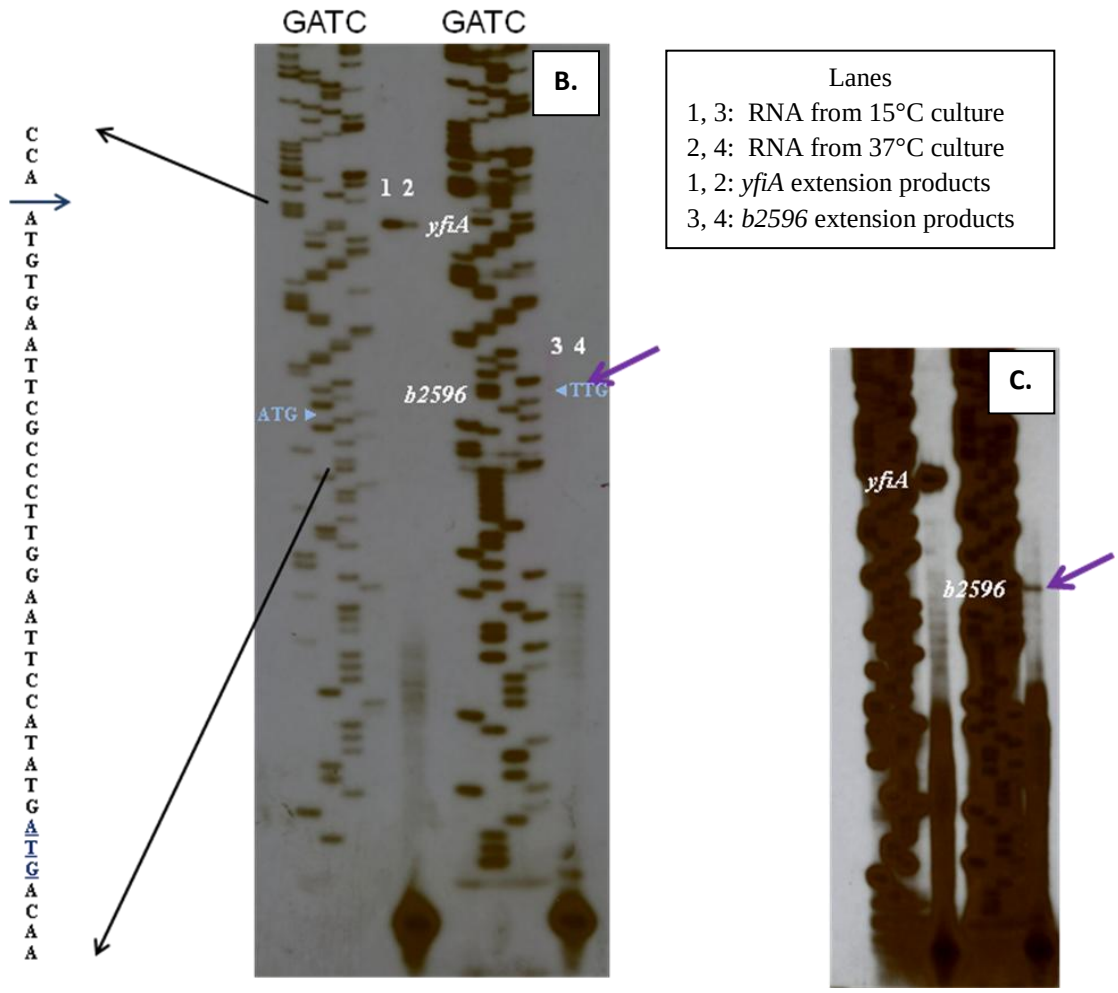


Figure 10: Transcriptional Start Site Analyses of *b2596* and *yfiA*
B.: Primer extension analyses to determine transcriptional start sites for *b2596* and *yfiA*. The transcriptional start site for *yfiA* is 29 base pairs upstream of the ATG start codon, indicated by the **blue** arrow. The *b2596* transcript begins at the TTG start codon, indicated by the purple arrow. **C**. Darker exposure of the primer extension gel to show *b2596* extension product observed in lane 3; indicated by the purple arrow in B and C.

It is interesting to observe that we detected a *b2596* transcript only in lane 3; the RNA sample extracted after exposing cells to cold-shock. We can infer that *b2596* mRNA levels in cells at 37°C are significantly lower than those at 15°C. This is in accordance with the data obtained while testing *b2596* mRNA expression.

Protein Expression and Purification of *b2596* (PX) and *yfiA* (PY)

It is characteristic of TA toxin and antitoxin to interact to form a stable complex. In order to test this tenet we needed independent expression of PX and PY. To visualize the protein product of *b2596* (Protein X) and *yfiA* (Protein Y) we expressed both genes in different vectors at 37°C and 15°C for 6 to 24 hours. Table 6 lists all the different vectors that *b2596* expression was attempted in. We tried expression with *b2596*-pColdI, *b2596*-pET28, *b2596*(ATG)-pColdI and *b2596*(ATG)-pET28. We also tried purifying the protein utilizing the $(His)_6$ affinity tags that were placed on *b2596* in our constructs. We also tried to visualize the protein using Western Blot Analyses with the Universal His Western Blot Kit. We were unable to detect PX expression until we constructed a *b2596*-pCold-TF clone. As expected, the PX-TF band migrated just above the trigger factor band and was only detectable 4 hours post induction (Figure 11A). Once we confirmed expression, we sought to obtain large amounts of the PX-TF fusion protein. For this we expressed *b2596*-pCold-TF in BL-21(DE3) cells for 6 hours and isolated it after Ni-NTA affinity chromatography. In this purification strategy, Trigger factor has a $(His)_6$ tag, thus the PX-TF fusion protein binds to the purification column. Results from this purification assay are shown in Figure 11B.

In contrast to PX, we had no difficulty expressing and purifying PY. PY was expressed in *yfiA*-pET21c and *yfiA*-pColdIV at 15°C in BL-21(DE3) and extracted from the cells. The *yfiA*-pET21c vector expressed PY with a $(His)_6$ tag and purified by Ni-NTA affinity chromatography (Figure 12). PY migrated slightly below the 14.3 kDa marker, consistent with its molecular mass of 12.7 kDa.

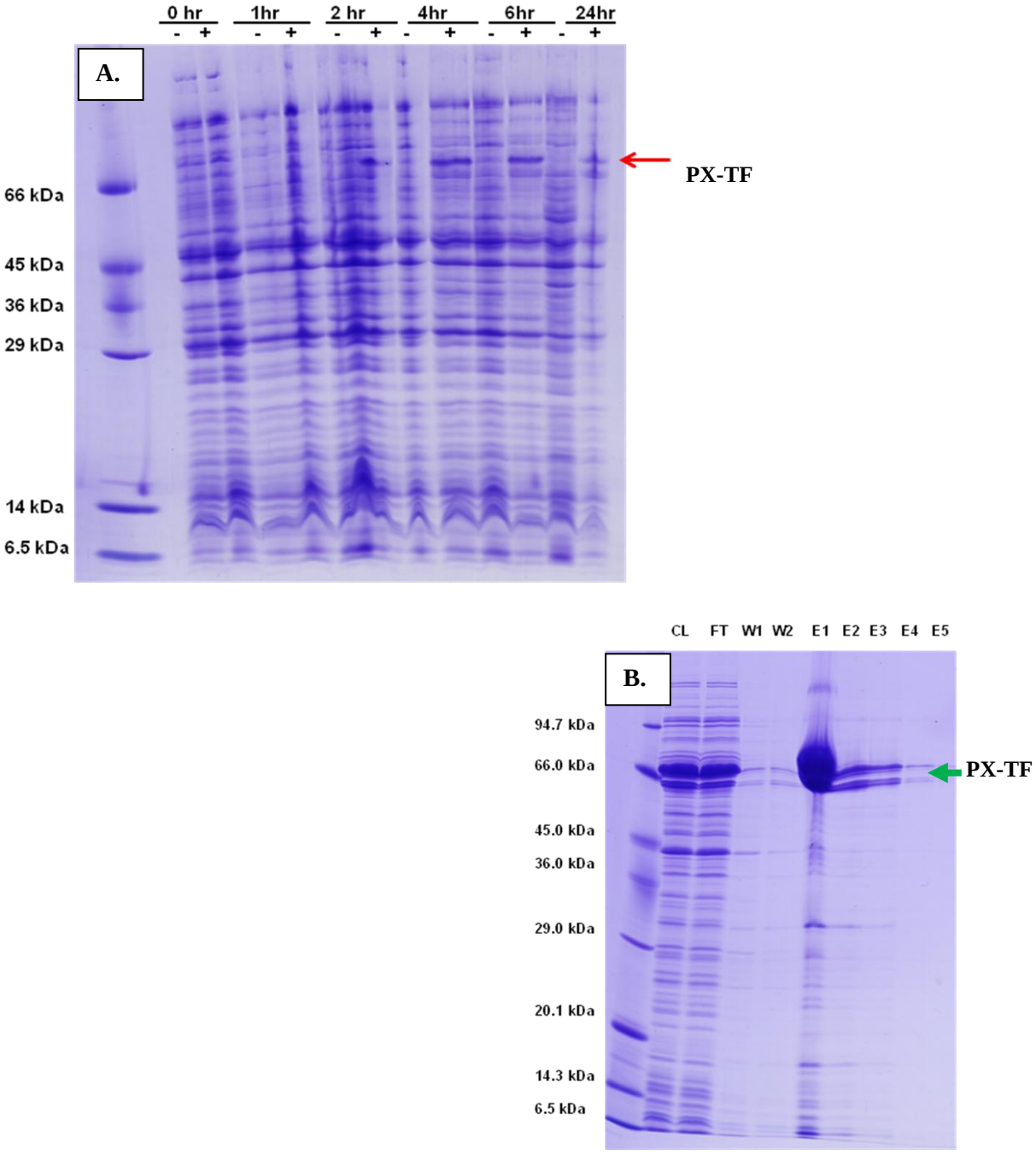


Figure 11: Protein X-Trigger Factor Expression & Purification
A. Expression of B2596 (PX) in *b2596*-pCold-TF in BL-21(DE3) cells: Time points were taken 0, 1, 2, 4, 6 and 24 hours post induction from the induced (+) and uninduced (-) cultures. The red arrow indicates the PX-TF band seen in the induced samples of the 4, 6 and 24 hour time points. **B. PX-TF purification:** PX-TF expressed for 6 hours in *b2596*-pCold-TF in BL-21(DE3) cells and purified over a Ni-NTA column. The lanes are: CL; Clear cell lysate, FT: Flow through, W1, W2: Washes and E1-E5: Elutions. The green arrow indicates PX-TF.

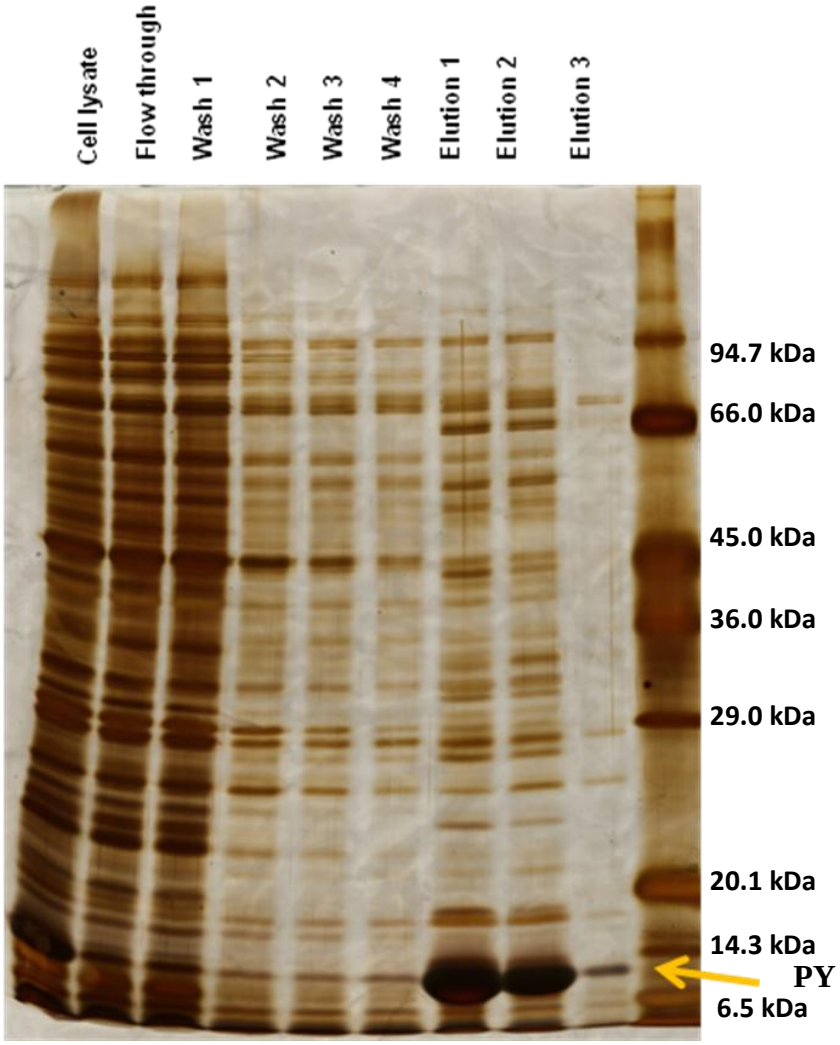


Figure 12: Expression of Protein Y in *yfiA*-pET21c: Protein Y, $(His)_6$ tagged, was expressed in BL-21(DE3) cells for 24 hours post induction and purified. A 17.5% SDS-PAGE gel was run and silver stained to visualize the elution fractions. The yellow arrow shows PY in Elutions 1, 2 and 3.

Northern Blot Analyses

Our primer extension data demonstrated that *b2596* and *yfiA* have independent transcriptional start sites. Since this result goes against the orthodoxy of TA modules we wanted to confirm the data using a different approach. To this end we performed three different Northern Blot analyses on total RNA extracts from BW25113 cells grown at 37°C and 15°C.

In order to estimate the size of the *yfiA* transcript, we hybridized the Northern Blot with an oligonucleotide that annealed directly upstream of the *yfiA* start codon (Green arrow, Figure 5). Primer NWO956, which hybridized to the *yfiA* coding region, was used as a positive control (Blue arrow, Figure 5). We saw bands slightly below the 0.5 kb marker and the of the RNA sample extracted after exposing the cells to cold-shock were markedly stronger (Figure 13A).

Next, we constructed a DNA fragment spanning the entire length of *b2596*. No visible bands were detected by Northern analysis with this fragment, even with long exposure periods. We then hybridized the same membrane with a DNA fragment spanning the entire length of *yfiA* (Figure 13B). In the third set of Northern Blots we designed a DNA fragment that would bind to a partial region of *b2596* (made through PCR using primer NWO961 and NWO958). We transferred 15°C and 37°C RNA samples on to two membranes in an identical pattern. We hybridized one membrane with the partial-*b2596* fragment (orange dashed line, Figure 5) and the other with a complete-*yfiA* fragment. The partial-*b2596* fragment did not reveal any bands. Figure 13**C** shows the results for the second hybridization.

For all three Northern blot analyses we detected bands just below the 0.5 kb marker. However, *yfiA* is 342 bases in length and has a 29 base predicted 5'UTR. We also found a strong rho-independent termination signal comprising a 10 base pair G-C rich hairpin loop and a U-rich tail 23 base pairs downstream of the *yfiA* stop codon. Thus the total length of the *yfiA* transcript is estimated at 422 bases. The migration of the *yfiA* RNA seen is consistent with the length of the *yfiA* transcript. Also we observed more signal for the RNA samples extracted after acclimating the cells at 15°C for 30 minutes in concordance with the role of *yfiA* in cold-shock.

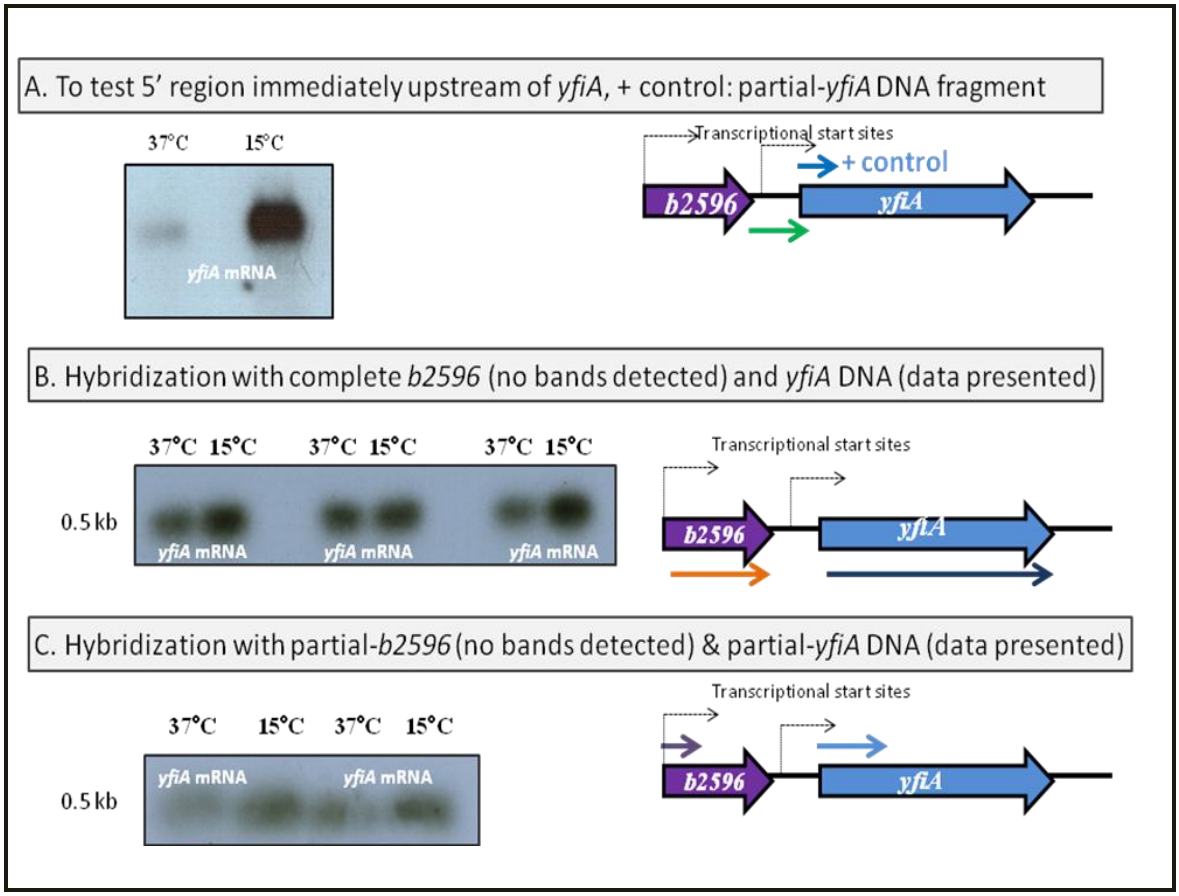
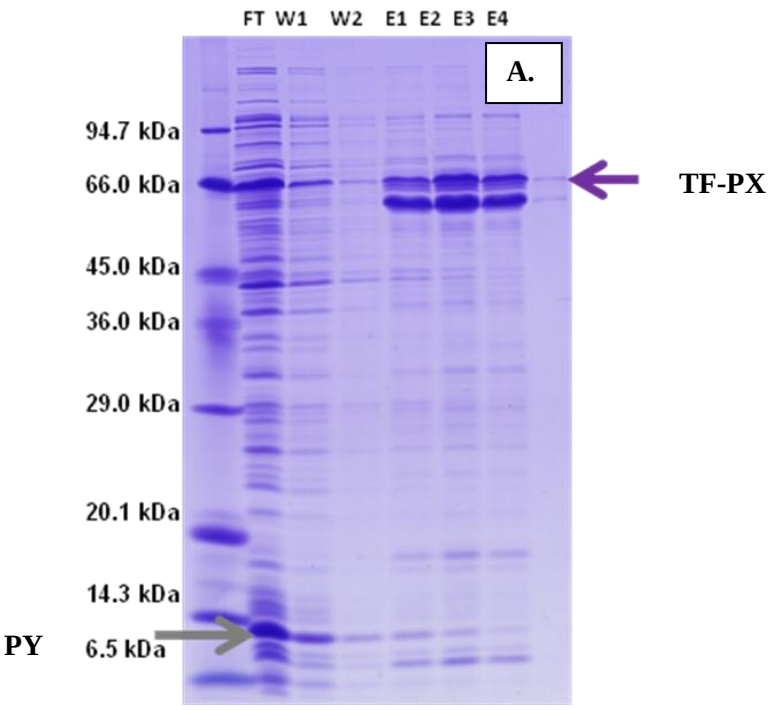


Figure 13: Northern Blot Analyses: **A**. **Green** arrow indicates test oligonucleotide. **B.** Two identical blots generated. No bands detected in blot hybridized with *b2596* DNA (**orange** arrow), data not shown. **C.** Two identical blots generated. No bands detected in blot hybridized with partial *b2596* DNA (**purple** arrow), data not shown. For all Northern blots we observed a fragment that ran close to the 0.5 kb marker.

Protein X and Protein Y Interaction Studies

Toxin and antitoxin products of canonical TA modules interact to form a stable complex. Therefore we investigated interaction between PX and PY. A mix of purified TF-PX and purified PY, along with Ni-NTA resin, was incubated at 4°C for 1hour to allow for interaction between the two proteins. This mix was then applied to a Ni-NTA column and elution fractions were collected and visualized on a protein gel. No interaction was observed (Figure 14A). However, we have encountered many instances where TF fusion to other proteins (TA toxins) inhibits their activity. It has been speculated that TF may sequester the small proteins fused to it or preclude the formation of the proper TA toxin stoichiometry required for activity.

We then tested interaction between PY and PX was cleaved off of the TF-PX fusion protein using thrombin cleavage. The mix containing PX and TF was applied to a Ni-NTA column with resin to obtain pure PX (in the flow through) as TF remained bound to the resin via its $(His)_6$ tag. Interaction was tested between PX and PY as described above (Figure 14B). Unfortunately, we were unable to detect interaction between PX and PY using this approach as well.



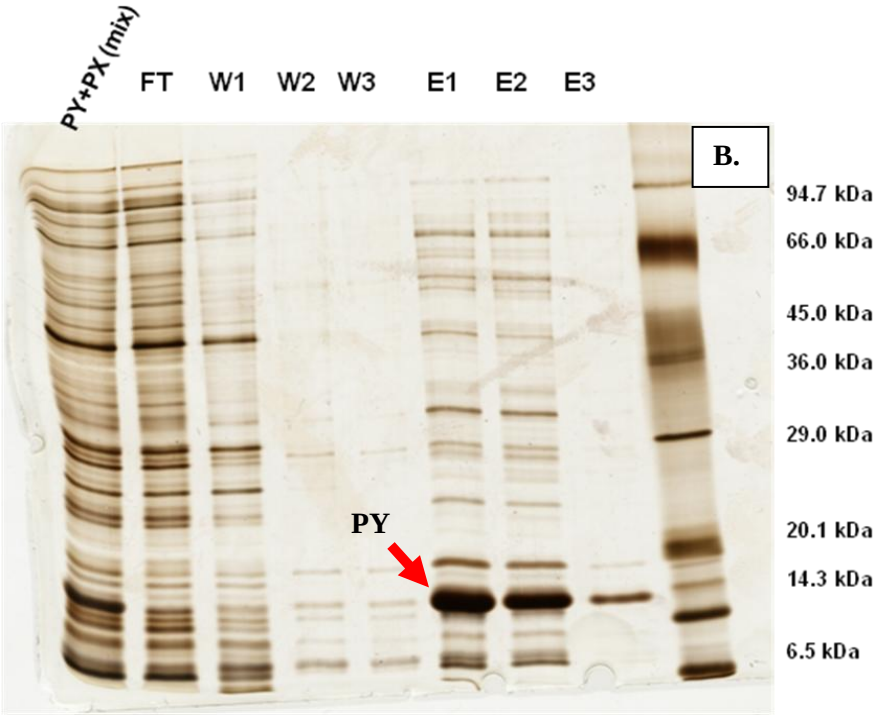


Figure 14: Protein X and Protein Y Interaction Studies: A. Interaction test between TF-PX and PY. PY, **grey** arrow, can be seen in the flow through sample (FT) and TF-PX in the elution fractions (E1-E4), **purple** arrow. **B**. Interaction test between PY$(His)_6$ and PX. PY is indicated in the elution fractions with the **red** arrow.

Protein X and Protein Y Growth Profiles

Since a TA system requires that one gene product be toxic and the other to counteract this toxicity, we tested toxicity of both *b2596* and *yfiA* over-expression. We grew *E. coli* cells harboring *b2596*-pColdIV or *yfiA*-pColdIV to mid logarithmic phase, shifted the cultures to 15°C to acclimate the cells for 30 minutes and then split the cultures and induced one half of each set with IPTG. As expected we saw that PX has no toxic effect (Figure 15A) and PY, the predicted toxin, when over-expressed causes a partial inhibition of cell growth when compared to the uninduced control (Figure 15B).

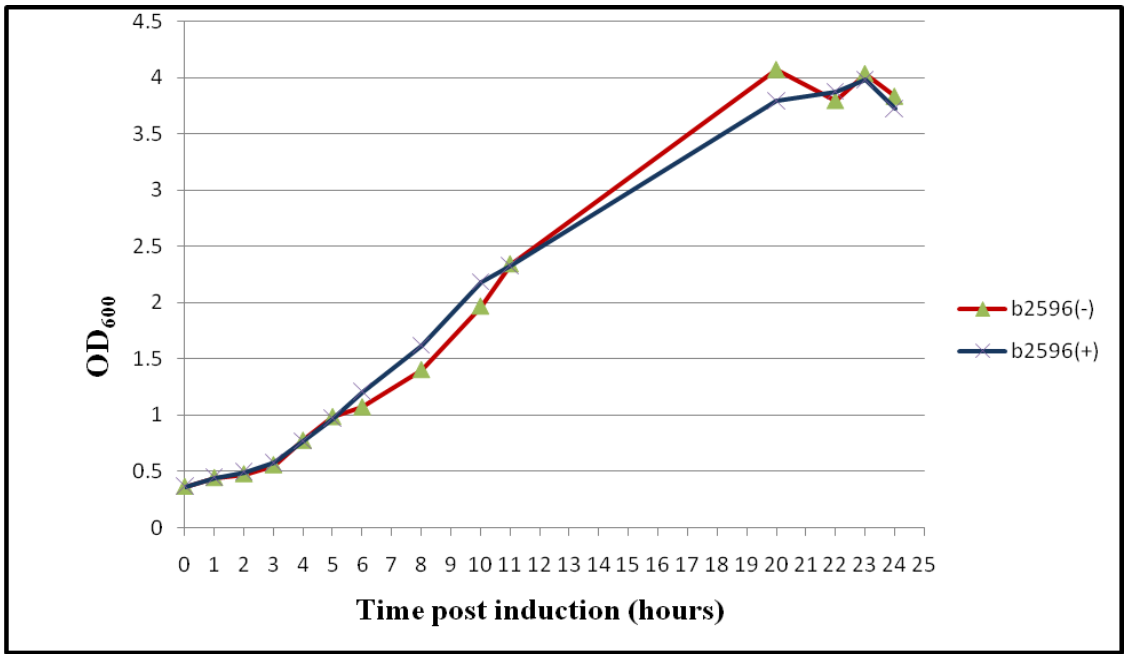


Figure 15A: Protein X and Protein Y Growth Profiles. BL-21(DE3) cells harboring *b2596*-pColdIV were grown to OD_{600}=0.3-0.4, shifted to 15°C and induced with 1mM IPTG (**Blue** line) or an equal volume of water as a control (**red** line). Time points were taken for a period of 24 hours and the results plotted on a linear graph. *b2596* shows no toxic effect.

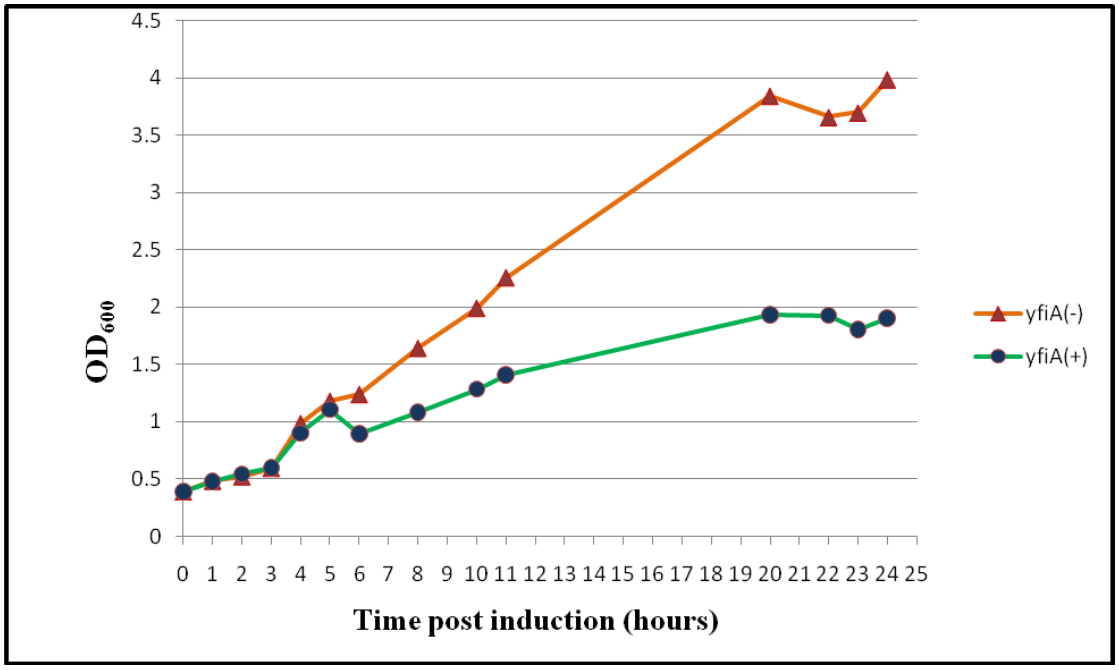


Figure 15B: Protein X and Protein Y Growth Profiles. BL-21(DE3) cells harboring *yfiA*-pColdIV were grown to OD_{600} =0.3-0.4, shifted to 15°C and induced with 1mM IPTG (**Blue** line) or an equal volume of water as a control (**red** line). Time points were taken for a period of 24 hours and the results plotted on a linear graph. *yfiA* shows toxic effect; inhibits growth by half.

The data shown for *b2596* is in concordance with the trend we documented for induced and uninduced samples of BL-21(DE3) cells harboring pCold-TF-*b2596* [data not shown], where no effect on cell growth was observed upon PX expression.

Discussion

PY associates with ribosomes and interferes with elongation by blocking the ribosomal A site and also decreases the overall accuracy of translation. [Agafonov et al., 2001; Maki et al., 2000; Vila-Sanjurjo et al., 2004]. This blocks translation and causes growth to slow down. Thus PY functions like a TA toxin. Our search for the cognate antitoxin gene revealed a small hypothetical ORF upstream of *yfiA*, designated *b2596*. We discovered that *b2596* and *yfiA* have opposite charges, encode small proteins and show sequence similarity to known TA system genes. Our initial investigation demonstrated that *b2596-yfiA* exhibits properties characteristic to TA modules (Figure 16).

All known TA systems genes are within one operon. However, our primer extension analyses showed that both genes have independent transcriptional start sites. The transcriptional start site for *b2596* coincided with the UUG start codon of the gene and that for *yfiA* was 29 bases upstream of the ATG start codon (Figure 10). We thus analyzed the upstream region of their transcriptional start sites in search of promoter sequences. We found a putative -10 and -35 region upstream the transcriptional start sites of both genes, showing matches to the consensus sequence for an ideal promoter. The consensus sequence for the -35 element is TTGACA and that for the -10 element, the Pribnow-Schaller Box [Pribnow, 1975; Schaller et al., 1975], is TATAAT. We found a perfect -35 sequence 29 base upstream of the predicted *yfiA* transcriptional start site and a sequence matching the -10 element consensus, with two mismatches, 13 base pairs upstream of *yfiA*'s transcriptional start site. For *b2596* the suggested -35 region is located 33 bases upstream to the transcriptional start site and the Pribnow-Schaller box match

was 14 bases upstream of the transcriptional start site (Figure 8). Both showed a two base mismatch with their respective consensus sequences. This data supports the observation that *yfiA* is transcribed independent of *b2596*. The putative promoter regions upstream of *b2596* are highly degenerate relative to the known consensus sequences.

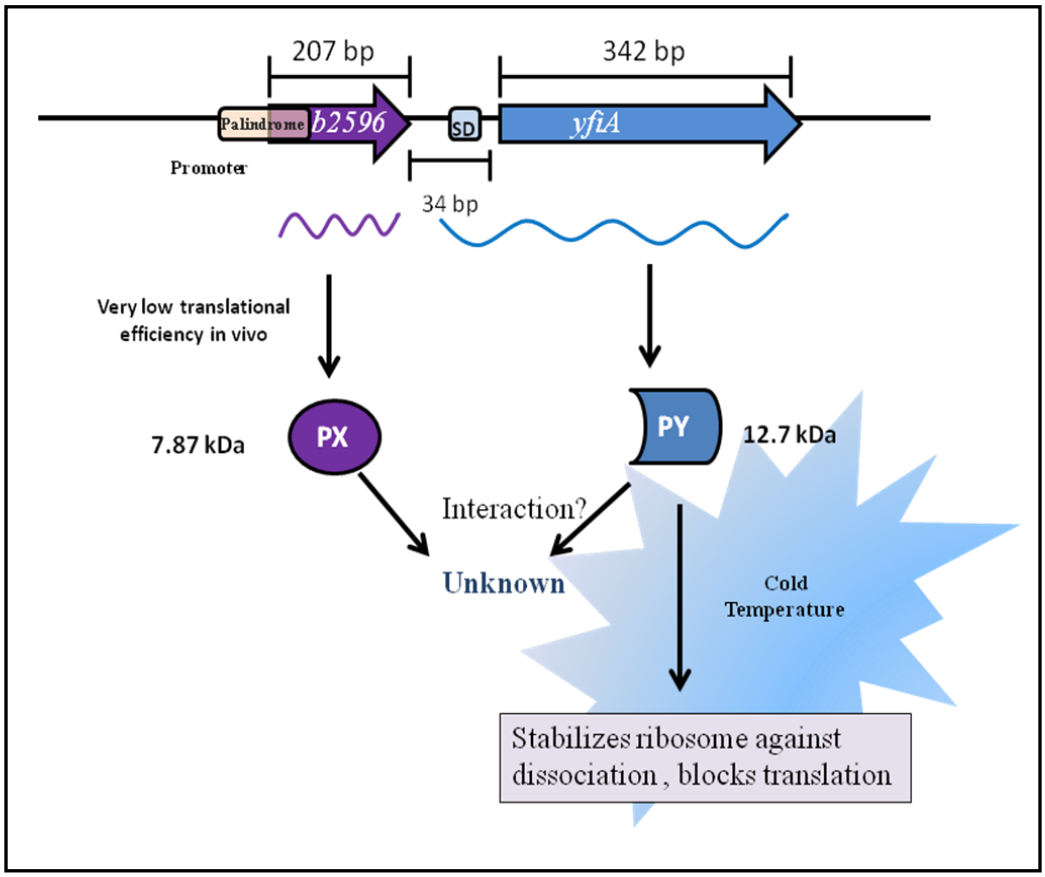


Figure 16: Features of *b2596-yfiA* module. 1. Gene positions: antitoxin precedes the toxin. **2**. Both genes encode small proteins and these proteins have opposite charges; PX is 68 amino acids in length with a pI of 11.3 (basic), PY is 114 amino acids in length with a pI of 6.57 (acidic). **3**. Palindrome: 28 base pairs, spanning the upstream and coding region of *b2596* is suggested. **4**. Both genes have independent transcriptional start sites and promoter regions to assist transcription. **5**. A Shine Dalgarno (SD) sequence was found upstream of the *yfiA* start codon. **6**. PY, proposed toxin, is observed in cold-shock conditions, and stationary phase, and inhibits translation.

b2596 encodes a leaderless mRNA

Our transcriptional start site analysis indicated that the *b2596* transcript begins at the TTG start codon of the gene. This indicated that *b2596* encodes a leaderless mRNA. Since leaderless transcripts lack a 5'UTR only the start codons contribute to transcript association with the ribosome through direct basepairing with initiator tRNA at the ribosomal P site [Jones et al., 1992]. In leaderless mRNAs different start codons contribute varyingly to mRNA stability and also influence translational efficiency. The AUG start codon is needed for efficient binding of 30S ribosomal subunits to leaderless mRNA in vitro [O'Donnell & Janssen, 2001]. Changing the AUG start codon to GUG resulted in a 12-fold reduction in expression whereas changing it to a UUG (as is the case with *b2596*) or CUG reduced expression to background levels [O'Donnell & Janssen, 2001]. This is possibly because wobble destabilizes the transcript-ribosome-tRNA complex, but this has not been shown in vivo [Moll et al., 2002; O'Donnell & Janssen, 2001]. These studies predict that there may be low of no in vivo translation of *b2596* due to the fact that it has a leaderless mRNA transcript that relies on a UUG start codon. There is also evidence showing that at low temperatures leaderless mRNAs are translated better than those containing an internal ribosome binding site, whereas at higher temperatures (42°C) translational efficiency of canonical mRNAs is superior by far [Grill et al., 2002].

Palindrome and Promoter Sequences:

All TA modules known so far have the ability to bind a palindromic sequence upstream of the antitoxin start site as a means of exerting autoregulatory feedback [Engelberg-Kulka and Glase, 1999; Gerdes et al., 2005]. Interestingly we also found a 28 base pair palindrome, with one mismatch, spanning the immediate upstream region of *b2596* and into the *b2596* coding region. The suggested -10 element of *b2596* overlapped with this palindrome as indicated in Figure 8. This palindrome could prove to be the autoregulatory sequence for the original *b2596-yfiA* module. Since our data indicates that *b2596* only translated at low levels in vivo this palindrome still exists, almost perfect, and may not be utilized for autoregulation. Next we located a sequence upstream of the *yfiA* ATG start codon that matched the Shine-Dalgarno consensus sequence, albeit missing one base. Thus *yfiA* has a promoter that helps its translation, however no such sequence was observed for *b2596*, strengthening the data that *b2596* may not be translated in vivo.

Functional Interaction between PX and PY

There are some aspects of the *b2596-yfiA* module that need further investigation to help characterize *b2596-yfiA* as a novel TA module. One such crucial piece is generating evidence to show that PX and PY directly interact, at least in vitro. However, we were unable to show interaction between the gene products of *b2596* and *yfiA* (Figure 14) even though interaction between the two gene products is one of the central tenets of TA module characteristics. We have not completed our interaction analyses and so cannot confirm or exclude interaction at this time.

YfiA and its 'toxicity'

One main characteristic of known TA modules is that the toxin product, when over expressed, causes growth arrest in bacterial cells. ProteinY associates with ribosomes upon the induction of cold-shock, as well as during stationary phase, stabilizing the monosomes against dissociation [Agafonov et al., 2001; Maki et al., 2000]. PY binds to the ribosome at the interface of the 50S and 30S subunits through specific interaction with the 30S subunit and prevents dissociation of the ribosome [Agafonov et al., 2001; Maki et al., 2000]. When normal growth conditions recommence and the environment returns to physiological temperature (37°C) PY's affinity for the ribosome diminishes and translation resumes. Thus PY does act as would be expected of a toxin of Toxin-Antitoxin system. Our growth assays show that on induction of PY, cell growth was reduced by half or more (Figure 15).

Predicted size of yfiA transcript

To convince ourselves that the bands observed by Northern analyses were those of *yfiA* mRNA we studied the sequence of the region upstream and downstream of *yfiA*. We found a typical rho-independent hair pin in the downstream region and therefore predict that the transcriptional termination start site is approximately 51 base downstream of the *yfiA* stop codon (Figure 17)

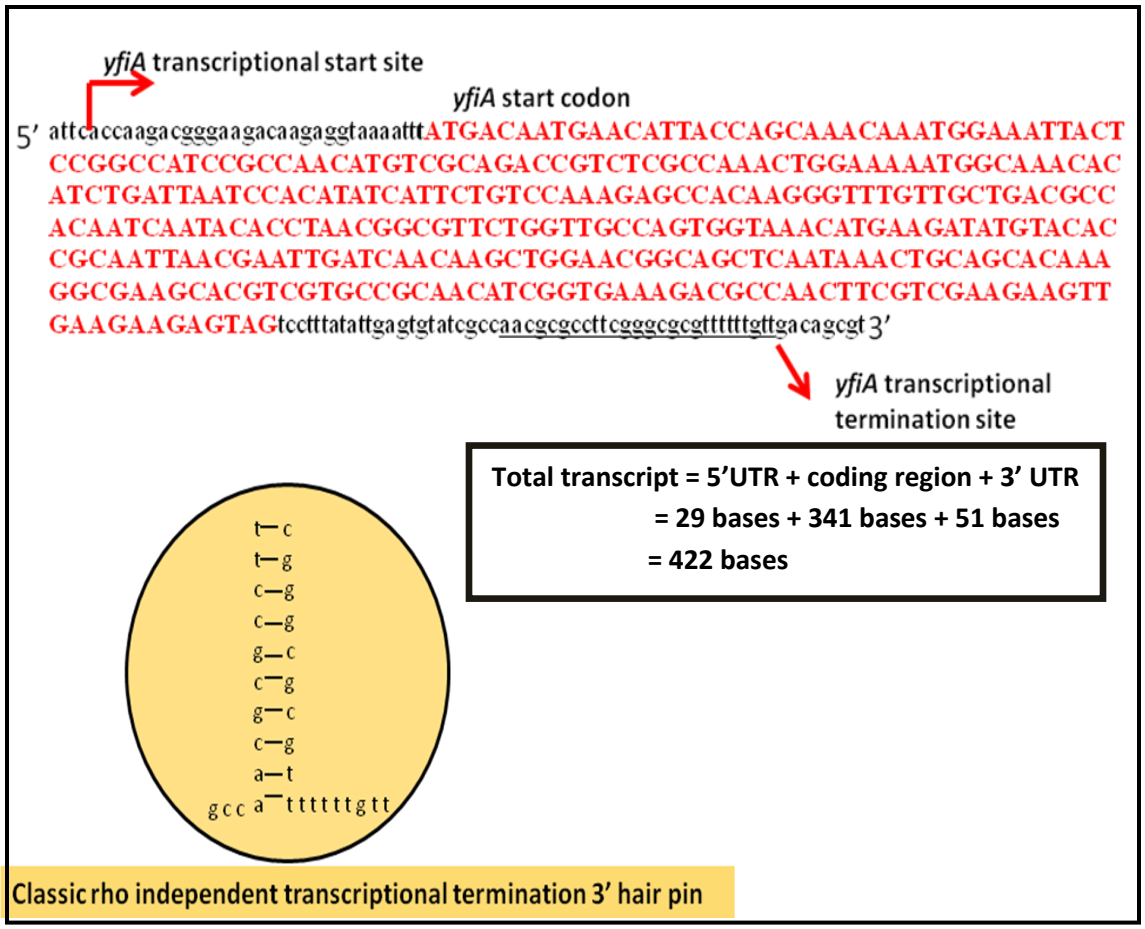


Figure 17: Predicted size of *yfiA* transcript: A classic rho-independent transcriptional termination 3' hair pin sequence. The size of the total transcript was predicted to be 422 bases.

Regulation of *yfiA*

TA systems are autoregulated by a feedback mechanism as described earlier. We observed a potential autoregulatory sequence, a 28 base palindrome, upstream of and in the coding region if *b2596*. We also found that *b2596* is transcribed only during cold shock and at very low levels. This suggests a possible role of the palindrome in cold shock associated regulation. We have not studied whether PX or the PX-PY complex binds to the palindromic region to exert autoregulation. If this palindrome is significant it does not function like a classic TA system autoregulatory sequence.

It has been proposed that three major cold shock proteins, CspA, CspB and CsdA, can be regulated at low temperatures by a highly conserved 11-base sequence, termed the "cold box", which exists in the 5'UTR [Fang et al., 1998]. Since *yfiA* functions like a cold shock protein, the presence of a cold-box was investigated and we did not find a sequence matching it. So what regulates *yfiA*? Answering this question will be crucial to decipher the *b2596-yfiA* story in totality.

Conclusion

We hypothesize that *b2596-yfiA* is a now-defunct TA module that has evolved from a canonical proteic TA system. Translation of *b2596* in vivo has not been seen and *yfiA* (the toxin of the TA module) shows significant levels only during cold-shock conditions, and during stationary phase. The original, functional *b2596-yfiA* TA module could possibly have been regulated by cold temperatures. The two genes are now independent. The regulator of *yfiA* at present is unknown. Figure 18 depicts our model of the evolution of *b2596* and *yfiA* from a canonical TA module possibly regulated by cold temperatures. *b2596-yfiA* no longer functions as a canonical TA system.

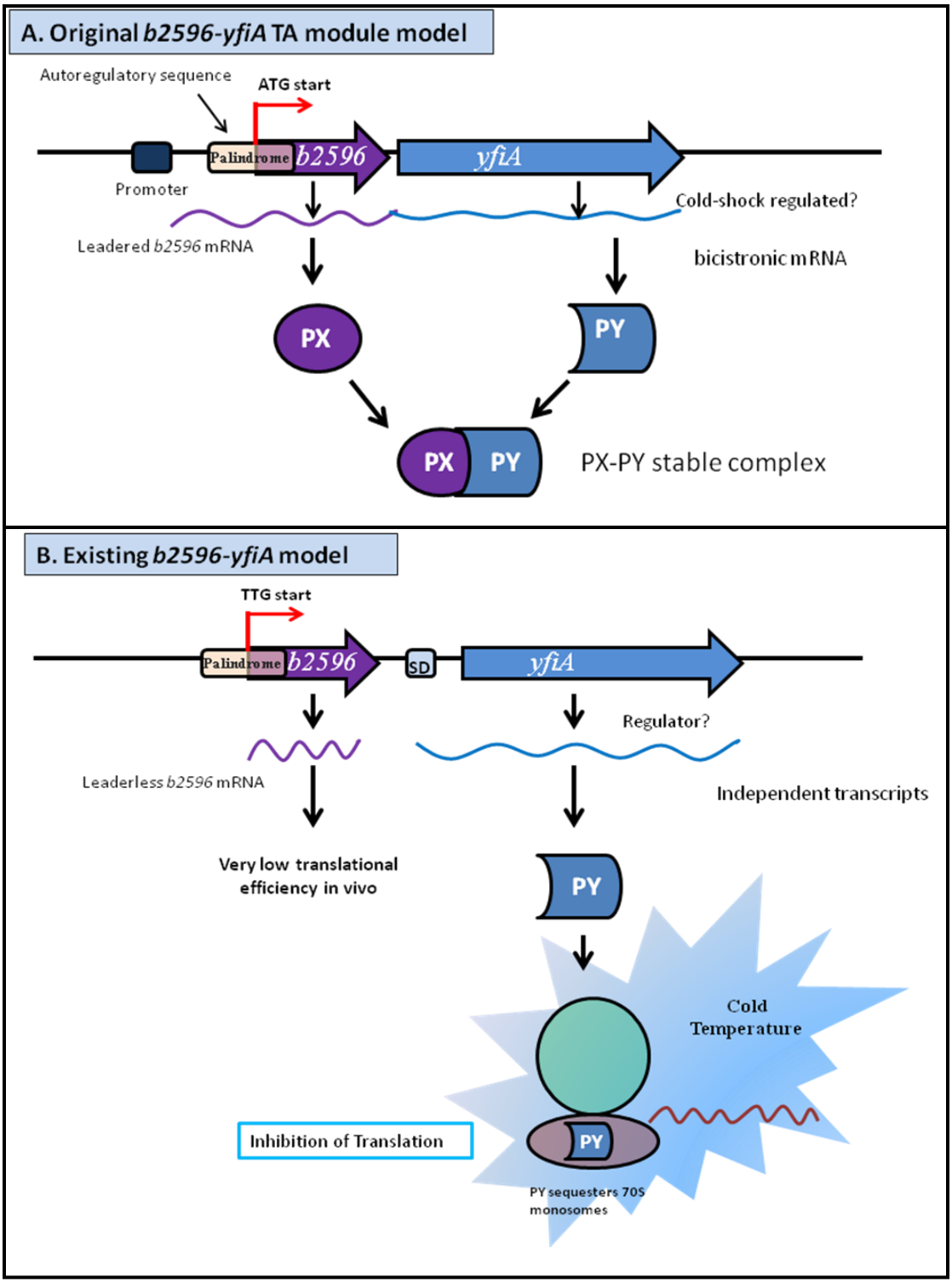


Figure 18: Original (predicted) *b2596-yfiA* TA module and existing *b2596* and *yfiA* system. A: Original, predicted *b2596-yfiA* TA module. *b2596* shown with ATG start, leadered mRNA, bicistronic mRNA for entire module, interaction between PX-PY. **B**: Existing *b2596* and *yfiA* gene properties.

Future Directions:

- Electrophoretic mobility shift assay (EMSA) to test whether PX-PY or either protein alone will bind the suggested palindromic sequence that is seen spanning the upstream region of *b2596,* going into the gene itself. This will determine whether the toxin-antitoxin complex has a autoregulatory role and will help to further characterize *yfiA* regulation.
- Interaction between the gene products of *b2596* and *yfiA* needs to be further studied. If *b2596-yfiA* was originally a true TA module that has now become defunct it is important to demonstrate interaction between PX and PY.
- We have not been able to identify the regulator for the *b2596-yfiA* module and suggest that cold shock be studied as a candidate for this. Since PY is only seen at low temperatures, dependency of this module on temperature, in the past or current, as a regulator seems plausible and should be investigated in vitro.
- Phylogenetic Analyses: To test whether *b2596* and *yfiA* are found in other species too and whether there is significant similarity or variation. This would also give us information on why *b2596* shows inefficient translation and whether the rare codons that we see in its sequence contribute largely to this.

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